CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

# THE EFFECT OF SULPHUR DIOXIDE ON PROBIOTIC AND PATHOGENIC BACTERIA OF THE HUMAN GASTROINTESTINAL TRACT

By
**Usman Ali Shah**

October 2019

CATÓLICA
ESCOLA SUPERIOR DE BIOTECNOLOGIA
PORTO

# THE EFFECT OF SULPHUR DIOXIDE ON PROBIOTIC AND PATHOGENIC BACTERIA OF THE HUMAN GASTROINTESTINAL TRACT

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Applied Microbiology

by

**Usman Ali Shah**

**Place: CBQF/Escola Superior de Biotecnologia da Univesidade Católica Portuguesa**

Supervision: Doutora Maria Manuela Pintado
Co-supervision: Doutor José António Couto
Doutora Ana Raquel Madureira

October 2019

**Abstract**

The major objective of the present research work was to determine the effect of sulphur dioxide ($SO_2$) on probiotic bacterial strains (*Lactobacillus acidophilus* KI, *Lactobacillus rhamnosus* R11, *Lactobacillus plantarum* , *Bifidobacterium animalis* Bo, and *Bifidobacterium animalis* Bb12) and pathogenic bacterial strains that could affect gastrointestinal system (GIT) (*Listeria monocytogenes*, *Escherichia coli, Salmonella enteritidis*, and *Bacillus cereus*) using an *in vitro* simulation model.

In a first step, growth curves were performed for both probiotic and pathogenic strains in culture media supplemented with $SO_2$ at 1000 mg/L and 500 mg/L and the optical densities were registered. It was observed that $SO_2$ at 500 mg/L did not cause significant reduction of any of the microorganism, however, the higher concentration of $SO_2$ (1000 mg/L) exhibited an inhibitory effect on *B. animalis* Bo. As for the pathogenic strains, only *L. monocytogenes* and in a less extent *E*. *coli* were inhibited when treated with $SO_2$ (1000 mg/L). The evaluation of the effect of $SO_2$ at 1000 mg/L on the viability of *B. animalis* Bo was further performed and a reduction of 1 log was registered.

When exposed to GIT conditions, the $SO_2$ showed to somewhat protect pathogenic strains from stomach conditions. At intestinal simulated conditions an inactivation effect of $SO_2$ at 1000 mg/L on *B. animalis* Bo was observed (1.9 log reduction). The other probiotic strains did not suffer a significant inactivation effect, in fact, it seems that are protected by the presence of $SO_2$.

Along the GIT simulation, the concentration of $SO_2$ did not change significantly, but according with the zeta potential the chemical form of the compound changes from sulphite ($SO_3^{2-}$) to bisulphites ($HSO_3^-$) and then to sulphur dioxide ($SO_2$), with the pH modulation from gastric to intestine conditions. Also, the metabolism of *B. animalis* Bo in the presence of $SO_2$ (1000 mg/L) is highly affected especially the glucose consumption. But no changes were observed in the production of organic acids such as acetic, and propionic acids, but lactic and citric acids were highly affected, succinic acid was somehow inhibited, but no production of butyric acid was observed.

As a conclusion, in general, $SO_2$ is not harmful for gut microbiota if ingested with food and it's a safe compound to use for food preservation. Nevertheless, if ingested jointly with contaminant bacteria or when finding these bacteria at gut, this compound don´t perform any antimicrobial effect.

**Key-words:** sulphur dioxide, probiotic bacteria, pathogenic bacteria, *in vitro* gastrointestinal tract

**Resumo**

O presente trabalho de investigação teve como principal objetivo determinar o efeito do dióxido de enxofre ($SO_2$) em estirpes bacterianas probióticas (*Lactobacillus acidophilus* KI, *Lactobacillus rhamnosus* R11, *Lactobacillus plantarum*, *Bifidobacterium animalis* Bo e *Bifidobacterium animalis* Bb12) e estirpes bacterianas patogénicas que podem afetar o sistema gastrointestinal (TGI) (*Listeria monocytogenes*, *Escherichia coli*, *Salmonella enteritidis* e *Bacillus cereus*) usando um modelo de simulação *in vitro*.

Num primeiro passo, foram realizadas curvas de crescimento de estirpes probióticas e patogénicas em meios de cultura suplementados com $SO_2$ a 1000 mg/L e 500 mg/L com base em medições de densidade óptica. Observou-se que o $SO_2$ a 500 mg/L não causou redução significativa de nenhum dos microrganismos, no entanto, a concentração de 1000 mg/L exibiu um efeito inibitório em *B. animalis* Bo. Quanto às estirpes patogénicas, apenas *L. monocytogenes* e, em menor extensão, *E. coli* foram inibidas quando tratadas com $SO_2$ (1000 mg/L). O efeito do $SO_2$ a 1000 mg/L na viabilidade de *B. animalis* Bo foi posteriormente avaliado tendo-se observado uma redução de 1 log.

No sistema gastrointestinal, o $SO_2$ mostrou um efeito de proteção das estirpes patogénicas em relação às condições estomacais. Nas condições simuladas do intestino, foi observado um efeito de inativação do $SO_2$ a 1000 mg/L em *B. animalis* Bo (redução de 1,9 log). As demais estirpes probióticas não sofreram efeito significativo de inativação, de facto, parece que são protegidos pela presença de $SO_2$.

Ao longo da simulação TGI, a concentração de $SO_2$ não se alterou significativamente, mas de acordo com o potencial zeta, a forma química do composto muda de sulfito ($SO_3^{2-}$) para bissulfitos ($HSO_3^-$) e depois para dióxido de enxofre ($SO_2$), com a mudança do pH das condições gástricas para intestinais. Além disso, o metabolismo de *B. animalis* Bo na presença de $SO_2$ (1000 mg/L) foi afetado, especialmente o consumo de glicose. Mas não foram observadas alterações na produção de ácidos orgânicos, como os ácidos acético e propiônico, mas os ácidos lático e cítrico foram altamente afetados, o ácido succínico foi de alguma forma inibido, mas não foi observada produção de ácido butírico.

Como conclusão, em geral, o $SO_2$ não é prejudicial para a microbiota intestinal nas doees testadas. Também se observou que se ingerido em conjunto com bactérias contaminantes ou ao encontrar essas bactérias no intestino, não exerce efeito antimicrobiano significativo.

**Palavras-chaves:** dióxido de enxofre, bactérias probióticas, bactérias patogénicas, trato gastrointestinal *in vitro*

**Dedication**

This thesis is dedicated to my entire family, my grandparents, my parents and my siblings. My father Mir Ahmad Shah, the most decent, simple, hardworking, sober, very kind hearted and loving father. My mom, the amazon, very sophisticated, loving, always much more caring after us. I also want to dedicate my thesis to my beloved teachers, from the time start of my educational journey until this present stage. The journey is still begun, and it will move on and would never stop for my entire life. May Allah bless you all with strong health and prosperous life, though some of my teachers already passed away, May Allah has his blessing and mercy upon their souls. Amen!

**If you are not willing to learn, no one can help you. If you are determined to learn, no one can stop you**

**Zig Zigla**

**Acknowledgements**

There are some of very precious great sayings; “God help those, who help themselves”. “Try try again”. “Virtuous is its own reward” and “pen is mightier than sword”. After all, and first of all, I am graciously thankful to my great parents, my mom and dad. Because of them, I came in to being and they have very imperative role in my life and my life came to a proper track because of their great effort. They steadfast and suffered long for make me a very good human being. Today, what I am, was not possible and you made me what I wished for and have reached to such position. I am really very grateful to both of you for rearing me up, for teaching me morality, ethics and sincerity, which are the stepping stone to success and then supported me at every step of my life, whether there were financially problems, or, I need your advice and help. You both have always graciously helped me and made me out from difficult and torrid situation. And I am also thankful to my uncle Nawab Shah for helped me out by their good advises.

I would like to acknowledge the most stunning institution *“Escola Superior de Biotecnologia”* who made it possible this journey for me, and made my dreams come true. Who offered me a lot of opportunities and splendid research area for fulfilling and achievement of my goal?

The most important person, whose name comes at first order, is my supervisor professora doutora Manuela Pintado, who always believed in me and in my research work and she always bound me with her best guidance and expertise and made my dream come true to be a scientist. I am also sincerely thankful to my co-supervisor Professor Doutor José António Couto and professora doutora Ana Raquel Madureira for their best guidance, advises and manipulating lab expertise and made all these possible for me.

I would like to say thank you to my classmates especially “Alejandro Canaza Jorges” for helping me out in such difficult time with his best lab expertise, skills and who always there stood beside me and also grateful for your immense guidance. Thanks to Carolina, Margarida, Ana, Patricia, Dandara, Daniela, and so on, if I forgot some of these names. And to my lab colleagues especially thankful to Sergio who always helped me, Glenise, Joana, Sara, Eduardo, Pedro the great and nice man, you always helped me out, Ana Madureira, Marta, Manuela and so on.

I am very grateful to Christina and as well Carla, for always helping me with HPLC experiments and always acknowledged me with your level best.

At the end, I would like to mention my cousin, as well my brother, Abdullah Khan and brother Musa Khan and Nadar Shah for your kind advises to step forward towards my goal. And am also thankful to Niaz Ali Khan, who helped me out with many problems.

**Contents**

## ★ LIST OF FIGURES ★

## ★ LIST OF TABLES ★

**List of abbreviations**

| | |
|---|---|
| EU | European Union |
| GRAS | Generally Recognized as Safe |
| DNA | Deoxyribonucleic Acid |
| ADP | Adenosine Diphosphate |
| SOX | Sulphite Oxidase |
| MRS | Man, Rogosa and Sharp |
| MHB | Muller Hinton Broth |
| TSB | Tryptone Soy Broth |
| FIA | Flow Injection Analysis |
| MIC | Minimum Inhibitory Concentration |
| $LD_{50}$ | Lethal Dose “50%” |
| HPLC | High Pressure Liquid Chromatography |
| UV-vis | Ultra Violet-visible |
| DLS | Dynamic Light scattering |
| GIT | Gastrointestinal tract |
| CFU | Colony Forming Unit |
| Ca. | counting approximately |
| g | gram |
| mg | milligram |
| L | liter |
| mL | milliliter |
| min | minutes |
| M | Molar |
| N | Normality |
| ppm | part per million |
| V/V | Volume/Volume |

## 1. Introduction

### 1.1 Sulphur dioxide ($SO_2$)

Sulphur dioxide in ancient times was used for fumigating the houses, and in Roman and Egyptians times was used for sanitizing wine vessels. Sulphur dioxide is used as a food preservative since 17$^{th}$ century produced by burning sulphur in the casks already filled with cider (Roberts and. McWeeny, 1972). Ancient cultures such as Greeks and Romans have been using sulphites to minimize and prevent spoilage and discolouration of foods, to sanitize wine vessels or as a purifier and disinfectant. Moreover, fermented products, such as beer and wine may also have naturally occurring sulphites, which are produced by yeasts during fermentation process (Garcia-Fuentes *et al*., 2015).

### 1.1 Role of sulphur dioxide ($SO_2$) in food

#### 1.2.1 Inhibition of non-enzymatic browning

In foods such as canned heat processed meat like pork roll, sausages, luncheon meat, chicken roll and canned vegetables and fruits like peas, beans, carrots, potatoes, cabbages, pears, $SO_2$ is usually added as sulphites ($SO_3^{2-}$) and metasulphites ($(S_2O_5)^{2-}$) forms, which are used to prevent non-enzymatic browning of food stuffs. The colour production occurs, because of the reactivity between amino groups ($-NH_2$) and active carbonyl (C=O) groups, which results in the formation of the insoluble dark coloured polymers (Roberts and McWeeny, 1972).

In addition, occurs the participation of intermediates such as reducing sugars, simple carbonyl compounds (aldehydes and ketones), which generate the most reactive intermediates regarding colour formation having α, β-saturated carbonyl and di-carbonyl compounds (Walker,1985 and Wedzicha, 1984). $SO_2$ has the ability to deactivate these intermediates firstly by the formation of hydroxy-sulphonate, when reacts with carbonyl groups, and secondly, by making it less reactive towards colour formation, when $SO_2$ reacts with C=C group of *α, β*-unsaturated carbonyl intermediates (Wedzicha, 1984).

Some practical examples of $SO_2$ effects includes prevention of oxidation of canned tomato sauce and maintenance of its bright colour, or the prevention of beer oxidative changes of flavour and undesirable fermentation (Roberts and. McWeeny, 1972). Also, in fruit juices and other non-fermented beverages sulphiting agents prevent non-enzymatic browning and in white wine, $SO_2$ is very helpful in stabilizing the colour by trapping acetaldehyde, and acting as an antioxidant (Walker,1985).

#### 1.2.2 Inhibition of enzymatic browning

It is also known that pH is a basic factor that mostly affects the enzymatic activity and promote enzymatic browning. The pH value in food maybe lowered by adding acids or by production of acids in fermentative processes. In addition, the enzymatic activity (e.g. proteases, pectinases and cellulose) is reduced by lowering the water activity of food, by dehydration and addition of salts and sugars (Roberts and. McWeeny, 1972).

Sulphiting agents play critical roles in the prevention of enzyme mediated spoilage of food. Sulphite reacts with important flavoprotein enzymes, such as glucose oxidase, D-amino acid, L-amino acid, glycolate, lactate oxidase and thiamine dehydrogenase. The prosthetic groups of haem in cytochromes and peroxidase also reacts with sulphite products, leading them to lose activity (Walker, 1985).

In plant derived foods, sulphite ions react with heat stable peroxidases due to their prosthetic group, the iron of haem, which inhibits its catalytic activity, favoured by hydrogen peroxidase. An example has shown in sterilized peas, in which $SO_2$ prevents the off-flavour formation, caused by deterioration of peroxidase enzyme (Wedzicha, 1984).

Overall, $SO_2$ inactivates some crucial enzymes in food such as ascorbic acid oxidase and lipoxygenase but, the mechanism is not yet completely understood (Wedzicha, 1984).

In pre-peeled potatoes, like sliced and chipped potatoes and some peeled and sliced apples and other fruits, sulphites are used to control of enzymatic browning, which are especially used for catering or bakery purpose (Walker, 1985). Likewise, $SO_2$ also prevents oxidative browning of fruits, for example, disintegration of colour on the cut surfaces of apple. Enzymatic oxidative browning may happen due to conversion of colourless phenolic compounds to coloured quinones (Freedman, 1980).

In apples and potatoes, enzymatic browning is especially concerned with the enzyme polyphenol oxidases, commonly produced on cut surfaces. $SO_2$ also plays prominent role by which, sulphite ions chemically react with quinone intermediates, to give rise to substituted o-diphenol. Due to the enzymatic browning, there is oxidation of monophenols or diphenols to o-quinones followed by the formation of high molecular weight coloured products due to the condensation reactions (Wedzicha, 1984).

In grapes must, the addition of $SO_2$ greatly inhibits the enzymatic browning of white wines. Sulphites are used to prevent discolouration, eventually occurring due to the action of enzyme tyrosinase (Walker, 1985).

**1.2.3 $SO_2$ as a reducing /antioxidant agent**

In minced meat, $SO_2$ in the form of sulphites or meta-bisulphites are used to prevent the grey discolouration of minced meat. The $SO_2$ prevents the oxidation of myoglobin to form brown met-myoglobin colour rather than freshly oxygenated red colour of minced meat (Roberts and McWeeny, 1972). Similarly, in comminute meat, sulphiting agents are used to inhibit the oxidation of myoglobin to met-myoglobin (Walker, 1985).

In fruits, vegetables and beer, sulphites are used to prevent the formation of off flavours due to the oxidation of oils and carotenoids of food and prevent oxidative changes in beer (Walker, 1985).

**1.3 Role of $SO_2$ in the control of microorganisms in food and antimicrobial mechanism**

In general, in fruits such as grapes, raspberries, gooseberries and cherries, the $SO_2$ is used to prevent the attack of *Botrytis, Cladosporium* and other moulds. $SO_2$ is applied to these fruits in solution form dissolved in water (Roberts and McWeeny, 1972).

In the manufacture of jams and fruits pulps there is also the addition up to 3000 mg/kg of $SO_2$, which helps in the prevention of microbial contamination and in long-term (months) retention of ascorbic acid. Moreover, in table grapes, $SO_2$ may also be helpful to prevent from fungal deterioration such as *Botrytis cinereal* (Walker, 1985).

In sausages, $SO_2$ is added as a sulphites or meta-bisulphites, which is effective in inhibiting the growth of moulds, yeasts and Salmonellae, when they are stored at room or at refrigerated temperatures. Additionally, sulphurous acid may inhibit the growth of Gram-negative rods such as *Escherichia coli* and *Pseudomonas* rather than the growth of Gram-positive rods such as lactobacilli (Roberts and McWeeny, 1972). Even though, in United Kingdom, for fresh sausages, the sulphiting agents may act as an

antimicrobial agent, particularly in controlling *Enterobacteriaceae* including pathogenic *Salmonellae* (Walker, 1985).

In acid pickles, $SO_2$ prevents the attack of moulds (Roberts and McWeeny, 1972). However, in acid fruit products, molecular $SO_2$ is determined with antimicrobial activity dependent upon pH value. Sulphiting agent's activity such as biocidal/biostatic on microorganisms is generally in order: Gram negative-bacteria > Gram-positive bacteria > moulds > yeasts (Walker, 1985).

In addition, $SO_2$ applied in grape must exhibit the growth inhibition effect upon undesirable acetic or lactic acid bacteria. In brewing process, $SO_2$ has selective anti-microbial activity (Walker, 1985). In wine process, $SO_2$ suppresses the growth of wild yeasts and microorganisms producing unwanted acetic acids and lactic acids and it allows wine yeasts to multiply (Freedman, 1980). In fruit juices and beverages, $SO_2$ has bacteriostatic effect against *Acetobacter* spp. and lactic acid bacteria having the effect at low pH, with the concentration of 100-200 ppm, and bactericidal at higher concentrations.

In British fresh sausages, the growth of *Salmonellae* and other *Enterobacteriaceae* was inhibited by $SO_2$, where the source of $SO_2$ was sodium-metabisulphites with the $SO_2$ concentration of 600 ppm (Jay, 1998).

In the preservation of sliced peach, sodium-metabisulphites ($Na_2S_2O_5$) solution is very crucial and may inactivate *Listeria monocytogenes* before their dehydration. Such salt may release $SO_2$ with the theoretical yield of 67.4% after their dissolution. Since free $SO_2$ does not bind to any sort of chemical compound such as sugar, enzymes, and because of its un-ionized nature, $SO_2$ can easily cross the cell membrane and disrupt its normal metabolic activity of the bacterial cell. However, it is important to highlight that $SO_2$ only has antimicrobial activity at low pH value. (DiPersio *et al.*, 2004). Sulphites give theoretical yield of $SO_2.H_2O$ in aqueous solutions. As the pH decreases, the proportion of $SO_2.H_2O$ increases and the concentration of bisulphites ($HSO_3^-$) ions decreases. Sulphites having maximum inhibitory effect, when the acid of $SO_2$ $H_2O$ is in undissociated form and pH is lower than 4.0. The antimicrobial activity of both $HSO_3^-$ and $SO_3^{2-}$ ions is lower than that of un-dissociated $SO_2$ $H_2O$. Carbonyl compounds present in food interact with sulphites, which reduces its activity. The concentration of free sulphites required to inhibit microorganism's growth at pH 7.0 was for *Salmonella* 15-109 μl/mL, *Escherichia coli* 50-195 μl, *Citrobacter freundii* 65-136 μl, *Yersinia enterocolitica* 67-98 μl, *Enterobacter agglomerans* μl 83-142, *Serratia marcescens* 190-241 μl and *Hafnia alvei* 200-241 μl. Because of their high reactive property, it is difficult to determine the exact antimicrobial mechanisms for sulphites, but the major target of their inhibition involves with the disruption of the cytoplasmic membrane, inactivation of DNA replication, protein synthesis, inactivation of membrane-bound or cytoplasmic enzymes or reaction with individual components in metabolic pathways and they also inhibit the solute active transport (Vijay and John, 2001, Hui and Evranuz, 2015). Bisulphite can also be helpful in destroying aflatoxins, for instance both aflatoxins $B_1$ and $B_2$ can be reduced in corns (Jay, 1998).

**1.4 Use of $SO_2$ in dehydration process**

In fruits and vegetables granules and powders (dehydrated) $SO_2$ is used to prevent favourable environment for non-enzymatic browning. Blanching is a process (vegetables soak in boiling water for 2 to 4 min prior to dehydration) take place with addition of sulphiting agents: 1) Prevent the enzymatic

browning such as; polyphenol oxidase to control the heat stable enzymatic activity 2) Prevent the oxidation of ascorbic acid (Walker, 1985).

**1.5 Role of Metabisulphite in the sensory properties**

In wheat flour products such as biscuits, crackers, frozen pizza and pastry doughs, sodium metabisulphite is used in baking industry for the preparation of flour dough by sulphitolysis of disuphide bonds in the gluten, which reduce the time processing and elasticity of the dough (Walker, 1985). Furthermore, in wheat flour dough and biscuits, sulphite ions act upon disulphide bond of the protein cross linked three-dimensional structure and break down the disulphide linkage (Wedzicha, 1984).

In wine, metabisulphite is added during serial decantation of must. $SO_2$ promotes the formation of the glycerol and it provokes the product with ably and smoothness. It also assists clarification of wine by precipitation of colloid fraction (Freedman, 1980).

**1.6 Range concentration of $SO_2$ for food product as a European (EU) regulation and legislation**

In European Union (EU), European Parliament and of the Council on food additives, the sulphiting agents are regulated by regulation 1333/2008 and according to such regulation, sulphiting agents are considered as food additives other than sweeteners or colorants. And, they are administered and identified by special E-number. The regulation 1333/2008 allow the sulphiting agents to be used in food products and with other food additives, enzymes, flavourings and nutrients. The established range is from 10 mg/kg to 10,000 mg/kg depending on the food OIV (2011). European commission regulation (EU) (November 2011) put forward their consideration with different standards of preservatives used in food to preserve them from spoilage and contamination with harmful bacteria and its deterioration. $SO_2$ is used in food as a preservative with its mainly usable form as a sulphur dioxide (molecular form) and sulphites salts.

Since 1959, sulphiting agents have been listed as generally recognized as safe (GRAS), when used in accordance with good manufacturing practice, except only not used in meat products or in foods which is a source of vitamin $B_1$ (Pizzoferrato *et al*., 1998). Moreover, the addition of sulphites to fruits and vegetables intended to be sold or served in raw form to the public was banned by revocation of the GRAS status. Besides that sulphiting agents present in a food product in quantity higher than 10 ppm must be declared in the label(Garcia-Fuentes *et al.,* 2015) due to the potential intolerance of sensitive consumers. "**Table 1.1** shows food products containing $SO_2$ as a food preservatives"

**Table 1.1 -** EU list of food products allowed to incorporate $SO_2$ and sulphites (E 220-228) with maximum level allowed and restrictions.

| Food | Maximum level (mg/L or mg/Kg) | Restrictions/Exceptions |
|---|---|---|
| **Fruit and vegetables** | | |
| **Unprocessed fruit and vegetables** | 10 | Only table grapes, fresh lychees (measured on edible parts) and blueberries |
| **Entire fresh fruit and vegetables** | 100 | Only vacuum-packed sweetcorn |
| **Peeled, cut and shredded fruit and vegetables** | 50 | Only peeled potatoes |
| | 300 | Only onion, garlic and shallot pulp |
| | 800 | Only horseradish pulp |
| **Frozen fruit and vegetables** | 50 | Only white vegetables including mushrooms and white pulses |
| | 30 | Only frozen and deep-frozen potatoes |
| **Processed fruit and vegetables** | | |
| **Dried fruit and vegetables** | 50 | Only dried coconut |
| | 50 | Only white vegetables, processed, including pulses |
| | 100 | Only dried mushrooms |
| | 150 | Only dried ginger |
| | 200 | Only dried tomatoes |
| | 400 | Only white vegetables, dried |
| | 500 | Only dried fruit and nuts in shell excluding dried apples, pears, bananas, apricots, peaches, grapes, prunes and figs |
| | 600 | Only dried apples and pears |
| | 1000 | Only dried bananas |
| | 2000 | Only dried apricots, peaches, grapes, prunes, and figs |

| Food | Maximum level (mg/L or mg/Kg) | Restrictions/Exceptions |
|---|---|---|
| **Fruit and vegetables in vinegar, oil, or brine** | 100 | Except olives and golden peppers in brine |
| | 500 | Only golden peppers in brine |
| **Canned or bottled fruit and vegetables** | 50 | Only white vegetables, including pulses |
| | 250 | Only bottled, sliced lemon |
| | 100 | Only bottled white heart cherries; vacuum-packed sweetcorn |
| | 50 | Only processed white vegetables and mushrooms |
| | 100 | Only rehydrated dried fruit and lychees, mostarda di frutta |
| | 300 | Only onion, garlic and shallot pulp |
| | 800 | Only horseradish pulp |
| **Jam, jellies and marmalades and similar products** | 800 | Only jellying fruit extract, liquid pectin for sale to the final consumer |
| | 100 | Only jams, jellies and marmalades made with sulphited fruit |
| **Processed potato products** | 400 | Only dehydrated potatoes products |
| **Other confectionery including breath freshening micro sweets** | 100 | Only candied, crystallized or glacé fruit, vegetables, angelica and citrus peel |
| | 100 | Only glucose syrup-based confectionery (carry over from the glucose syrup only) |
| **Decorations, coatings and fillings, fruit-based fillings** | 50 | Only glucose syrup-based confectionery (carry over from the glucose syrup only) |
| | 40 | Only toppings (syrups for pancakes, flavoured syrups for milkshakes and ice cream; similar products) |
| | 100 | Only fruit fillings for pastries |
| **Cereals and cereal products** | 30 | Only sago and pearl barley |
| **Whole, broken, or flaked grain** | | |

| Food | Maximum level (mg/L or mg/Kg) | Restrictions/Exceptions |
|---|---|---|
| **Starches** | 50 | Excluding starches in infant formulae, follow on formulae and processed cereal-based foods and baby foods |
| **Fine bakery wares** | 50 | Only dry biscuits |
| **Meat preparations** | 450 | Only breakfast sausages; Burger meat with a minimum vegetable and/or cereal content of 4 % mixed within the meat |
| | 450 | Only salsicha fresca, longaniza fresca, butifarra fresca |
| **Unprocessed molluscs and crustaceans** | 150 | Only fresh, frozen and deep-frozen crustaceans and cephalopods; crustaceans of the Penaeidae, Solenoceridae and Aristaeidae family up to 80 units |
| | 200 | Only crustaceans of the Penaeidae, Solenoceridae and Aristaeidae family between 80 and 120 units |
| | 300 | Only crustaceans of the Penaeidae, Solenoceridae and Aristaeidae over 120 units |
| **Processed fish and fishery products including molluscs and crustaceans** | 50 | Only cooked crustaceans and cephalopods |
| | 135 | Only cooked crustaceans of the Penaeidae, Solenoceridae and Aristaeidae family up to 80 units |
| | 180 | Only cooked crustaceans of the Penaeidae, Solenoceridae and Aristaeidae family up to 80 and 120 units |
| | 200 | Only dried salted fish of the “Gadidae” species |
| | 270 | Only cooked crustaceans of the Penaeidae, Solenoceridae and Aristaeidae family over 120 units |
| **Sugars, syrups, honey and table-top sweeteners** | 10 | Only sugars, except glucose syrup |
| | 20 | Only glucose syrup, whether dehydrated or not. |

| Food | Maximum level (mg/L or mg/Kg) | Restrictions/Exceptions |
|---|---|---|
| **Herbs, spices, seasonings**<br>**Herbs and spices** | 150 | Only cinnamon (Cinnamomum ceylanicum) |
| **Vinegars** | 170 | Only fermentation vinegar |
| **Mustard** | 250 | Excluding Dijon mustard |
| | 500 | Only Dijon mustard |
| **Protein products** | 200 | Only analogues of meat, fish, crustaceans and cephalopods |
| | 50 | Only gelatine |
| **Fruit juices and vegetable juices** | 2000 | Only concentrated grape juice for home wine-making |
| | 50 | Only orange, grapefruit, apple and pineapple juice for bulk dispensing in catering establishments |
| | 350 | Only lime and lemon juice |
| | 70 | Only grape juice, unfermented, for sacramental use |
| **Flavoured drinks** | 20 | Only carry over from concentrates in non-alcoholic flavoured drinks containing fruit juice |
| | 50 | Only non-alcoholic flavoured drinks containing at least 235 g/l glucose syrup |
| | 350 | Only concentrates based on fruit juice and containing not less than 2.5 % barley (barley water) |
| | 250 | Only other concentrates based on fruit juice or comminuted fruit; capilé, groselha |
| **Alcoholic beverages, including alcohol-free and low-alcohol counterparts**<br>**Beer and malt beverages** | 50 | Only beer with a second fermentation in the cask |
| **Wine and other products and alcohol-free counterparts** | 200 | Only alcohol-free |
| **Cider and Perry** | 200 | |

| Food | Maximum level (mg/L or mg/Kg) | Restrictions/Exceptions |
|---|---|---|
| **Fruit wine and made wine** | 200 | |
| | 260 | only made wine |
| **Spirit drinks** | 50 | Only distilled alcoholic beverages containing whole pears |
| **Ready-to-eat savouries and snacks Potato, cereal, flour or starch-based snacks** | 50 | Only cereal and potato-based snacks |
| **Processed nuts** | 50 | Only marinated nuts |

Source: Modified from European Commission Regulation (2011)

### 1.7 Toxicity of $SO_2$ and its impact upon human beings

A person can develop sensitivity to sulphites at any time in his lifespan, but some initial reactions are vague until a person has reached their forties and fifties, and the most sensitive patients have reacted to a dose of 5 mg of sodium bisulphite (Garcia-Fuentes *et al.,* 2015). Since 1977, in medical literature, several cases of sulphite-induced asthma have been reported and many more non-substantiated reports have been sent to the Food and Drug Administration. Bisulphites level above 0.1% leads to manifestations of toxicity that included growth retardation, clinical polyneuritis, spectacle eyes, bleached incisor teeth, brown uteri, atrophy of various viscera, calcified renal tubular casts, atrophy of bone marrow and bone, myocardial necrosis and fibrosis, and gastric squamous epithelial hyperplasia. These results have been doubted, because of the presence of diminishing levels of sulphite in diets and the probable destruction of thiamine in diet. The diet prepared with the accumulation of meta-bisulphites when stored at room temperature may cause the depletion of thiamine and by its prolonged storage of 3-4 months at room temperature, the diet would cause health ailment, such as chronic diarrhoea, which is irreversible even though supplemented by thiamine (Taylor *et al.,* 1986). Individuals, who are experiencing sensitivity towards asthma, and their average age is close 40 years, mostly women are pertaining sensitivity predominantly. Though, the report of those victims is not obvious regarding pre-school children, maybe they could be consuming fewer foods in their diets with high sulphite content (Lester, 1995).

The range of most of the sulphite-sensitive individuals, did react to ingested metabisulfite, which are occurring from 20 to 50 mg in concentration, and the level of consumption is not fixed to a single person, but they may vary broadly depending on individual pattern of ingestion. The sulphite sensitive reactivity is diverse and mild, and these occurrences may consist of dermatologic symptoms such as urticaria, angioedema, hives and pruritus, flushing, tingling and swelling. Furthermore, respiratory symptoms including dyspnoea, wheezing, bronchoconstriction and gastrointestinal symptoms such as nausea and stomach cramps. Moreover, less common, but more severe nonspecific signs and symptoms, such as hypotension, cyanosis, diaphoresis, shock and loss of consciousness had been reported (Lester, 1995).

Sulphites may be generated endogenously as well, because of the body normal processing system by sulphur-containing amino acids. Cysteine and methionine are those amino acids responsible to produce sulphites in the body normally, which mays maintain the level of endogenous sulphites at a very low level and steady state. A mitochondrial enzyme, sulphite oxidase, is to be believed to have prominent role for the maintenance of such level of sulphites in the body and by promoting their oxidation to sulphates, which is excreted in urine. (Lester, 1995, Til and Feron, 1992).

According to the free radical-mediated toxic effect of sulphites, they lead to the reduction of ATP by affecting mitochondria. In the dissected isolate rat brain mitochondria examinations, it was demonstrated that, mitochondria were the direct target, because, the oxidation of glutamate with phosphorylation of ADP was found to be significantly inhibited by sulphite in a dose dependent manner (Kocamaz *et al.,* 2012).

Exogenously, sulphites (1000 mg) are produced per day continuously during the normal processing of sulphur-containing amino acids within tissues. The oxidation of sulphite to sulphate is catalysed by SOX (sulphite oxidase enzyme) to protect the cell from its damaging effect. This enzyme is basically a molybdohemoprotein residing in the mitochondrial intermediate space (Kocamaz *et al.,* 2012).

The physiological importance of sulphite detoxification becomes obvious in case of the enzyme, sulphite oxidase deficiency, which is rare and severe inborn metabolic disorder inherited in an autosomal recessive disorder. Sulphite oxidase (SOX) deficiency in the main clinical manifestations is concerned with extreme neurological deformities and categorized by severe mental retardation, seizures, spastic quadriparesis, disclosed lenses, progressive destruction of brain tissues and early death. These clinical findings are put forward by elevated levels of sulphite and their related metabolites, such as S-sulfo-L-cysteine in plasma and urine. Thus, endogenous sulphite has highly neurotoxic effects which has been shown by strong evidence (Kocamaz *et al.,* 2012).

Regarding sulphites toxicity, in studies performed in several mammalian species, no evidence of chronic toxicity was identified. For these contradictory results, it might be the efficiency of sulphite detoxification in mammalian animal models. Although rats have been using as an experimental model for the evaluation of sulphite toxicity, they are not considered as a convenient model for humans, because, rats have higher rate level of sulphite oxidase (SOX) activity responses, as compared to that observed in humans, the rat liver having a 20-fold greater SOX activity than human liver (Kocamaz *et al.,* 2012).

The $LD_{50}$ (Lethal dose 50%) of free sulphites for the acute oral toxicity is account to be in the range of 1000-2000 mg/kg. While, for bound sulphites concerning acute toxicity is probably less and exceeds towards 5000 mg/kg for 3-deoxy-4-sulphohexoulase (Til and Feron, 1992).

The studies of mutagenicity induced by sulphites have been revealing that sulphites have the capability of inducing mutations in several organisms, namely in yeast, *Escherichia coli* and *Vicia faba*, but only at high concentrations and at low pH values. Even though, there is no proof for sulphite-induced mutagenesis in *in vivo* system or in *in vitro* in the Ames *Salmonella* test or in human tissue or cell cultures. In long term animal studies with rats, there was no evidence that sulphite had any carcinogenic effect. Furthermore, sulphite fed to mice at 1 and 2% with $K_2S_2O_5$ (potassium meta sulphite) in drinking water had not provoked the tumorigenic effect, but, somehow a minor increased incidence of mammary

adenocarcinomas was observed in sulphite oxidase-deficient rates in the session of feeding for five months with tungsten, but such increase was not statistically significant (Til and Feron, 1992).

The sensitivity of exogenous sulphite is higher than the manifestations of endogenous sulphite sensitivity. Furthermore, the ingested sulphites by the activity of gastric environment are converted to $SO_2$. So, after burping, $SO_2$ may be inhaled and cause bronchoconstriction in people with airway hyperreactivity (Lester, 1995).

According to the investigation of reproductive toxicology by sulphites, in sulphite oxidase-deficient rats, sulphite had not shown any side effects and reproductive malformation. Before mating, the rats were exposed for 3 weeks to the sulphite, and after mating, it was observed that pregnancy rates, gestational weight gain, preimplantation loss, resorbed and dead foetuses, litter size, fatal weights and malformations were unaffected by sulphite treatment (Taylor *et al.,* 1986).

### 1.8 Ionization of sulphites and free form of $SO_2$

Sulphites present in different forms, which is characterized by sulphite ion $SO_3^{2-}$. The term sulphites are commonly used to describe the oxo species of sulphur oxidation state that is comprised of ionic and non-ionic forms: $SO_2$, $HSO_3^-$, $SO_3^{2-}$ and $S_2O_5$ (Garcia-Fuentes *et al.,* 2015). Sulphites, when dissolved in water, sulphur dioxide exists in equilibrium forms within molecular form ($SO_2 \bullet H_2O$), bisulfited ions ($HSO_3^-$) and sulphite ions ($SO_3^{2-}$), which are dependent on pH (Fuglsang and Edwards, 2007). The schematic diagram and figure have been shown below, which consists of interchangeable forms of sulphur dioxide ($SO_2$).

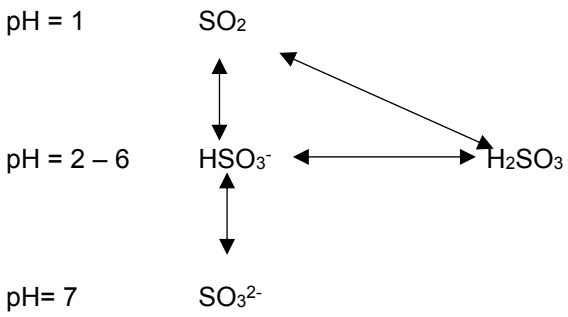



**Figure 1.1-** Sulphites chemistry in equilibrium state according Garcia-Fuentes *et al.,* (2015)

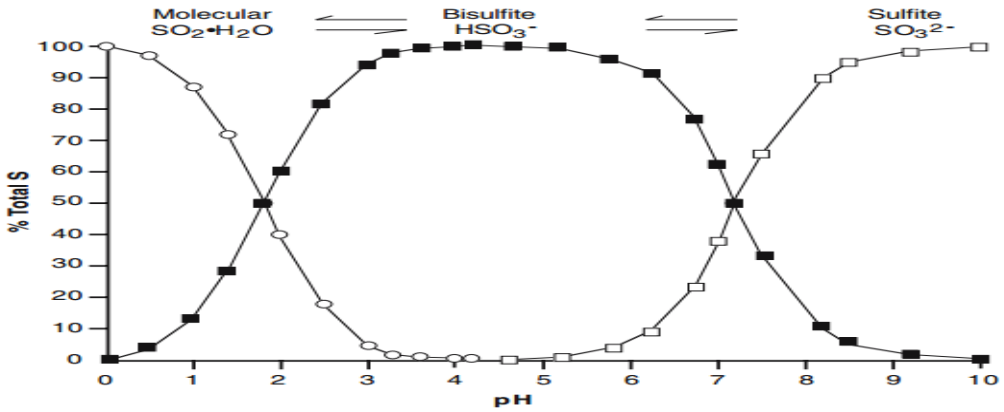



**Figure 1.2-** Presence of abounded level of molecular $SO_2$, bisulphite and sulphite at different pH values. (Source: Fugelsang, Kenneth C. and Edwards, Charles G. (2007)

### 1.9 Quantification of $SO_2$ in solution and food products

Several methods have been used to quantify sulphur dioxide ($SO_2$), in free and bound form within food products and beverages. The most optimized methods are Ripper method and aeration oxidation method. The first two methods deal with colorimetric titration. The ripper method is based on oxidation reduction reaction, which is mentioned below.

$$SO_2 + I_3 + H_2O \longrightarrow SO_3 + 3I^- + 2H^+$$

Ripper method induces the titration of free or total sulphur dioxide (bounded) of the sample with iodine, keeping the control final point of the titration with a starch indicator solution, which is already added to the sample. This method is more efficient for white wine. The determination of free sulphur dioxide is directly manipulated by such method, while for determining the total sulphur dioxide contents, the wine sample is first pre-treated with sodium hydroxide in order to release the bounded $SO_2$. Although, this is the most used method in the laboratories of wineries. But still, it has some inaccuracy in obtaining results. In red wine, because of complex matrix and having compounds like polyphenols, tannins and anthocyanins, they give false positive results with iodine despite of $SO_2$, which gives coloration (Pambianchi, 2014, Plaza *et al*., 2013, Luque de Castro, 2007)

Monier-Williams method is used to quantify total sulphites and sulphur dioxide in various food and beverages products. It is based on titration of $H_2SO_4$ solution with a base (NaOH) to a known endpoint. Initially, the wine sample is treated with phosphoric acid for their acidification in order to release $SO_2$. Stream of air has been passed through solution and free $SO_2$ has been collected, which then oxidized in a hydrogen peroxide ($H_2O_2$) solution to produce sulphuric acid ($H_2SO_4$) as the reaction given below.

$$SO_2 + H_2O_2 \longrightarrow SO_3 + H_2O \longrightarrow 2H^+ + SO_4^{2-}$$

The limitation of such method is to give false positive result because of ascorbic acid, oxidized to $H_2O_2$, which after may react with free $SO_2$, if excessive amount of acids is used (Pambianchi, 2014). Also, such method is laborious, time-consuming and shows false positive results (Chen *et al.,* 2015).

Another advantageous analytical method is flow injection analysis (FIA) that is used for the determination of sulphites in meat, food and beverages. Which is fast, precise, conclusive, accurate, versatile and with low cost and easy automation. FIA may also have concerned with small volumes of samples, low reagent consumption, minimum use of toxic substances and showing compatibility with almost any detection principle, using relatively simple instrumentation, and miniaturization possibilities. Such technique also shows flexibility for the routine determination of large numbers of samples (Ruiz-Capillas and Jimenez-Colmenero, 2008, Claudia and Francisco, 2009)

FIA is based on such system, in which the liquid sample is injected in to a floating, non-segmented continuous carrier stream of liquid, the injected sample forms a zone, then it is transported towards a detector, which has been continuously recording changes in their physical parameters, such as; absorbance or electrode potential, as the sample materials are flowing through the cell. The visualization of the FIA response curve (FIAgram) is the result of two kinetic processes and the dispersion of the

sample zone within carrier stream is due to physical process and the chemical process of formation of a chemical species. These two processes occur simultaneously and as well together with the dynamic characteristics of the detector, which yields the FIA response curve (Ruiz-Capillas and Jimenez-Colmenero, 2008).

There are some other methods also used for $SO_2$ determination in food such as titrimetry, electrochemistry, fluorimetry, chemiluminescence spectrometry, colorimetry, gas-liquid chromatography and liquid chromatography (Ruiz-Capillas and Jimenez-Colmenero, 2008, Claudia and Francisco, 2009). After all, HPLC (High Pressure Liquid Chromatography) is one of the easiest ways to manipulate and to analyse the free and total sulphite in vegetables and dried fruits using ultra violet-visible (UV-vis) detector (McFeeters and Barish, 2003).

**1.10 Objectives**

Considering that $SO_2$ is present in several food products, the question that remains is how this compound behaves during the passage by gastrointestinal tract conditions. Also, what would be the effect of $SO_2$ on probiotic and pathogenic bacteria of the human gastrointestinal tract. Hence, the first objective of this thesis was to optimize an *in vitro* simulated gastrointestinal tract model and evaluate the chemical and physical stability of $SO_2$ when exposed to the conditions prevailing in human GIT by quantifying and understanding the interactions with the gastric and intestinal juices components and monitoring the particle size and charges changes along the GIT.

Then, the second objective was to evaluate the effect of $SO_2$ on the viability of selected probiotic strains like, *Lactobacillus acidophilus* KI, *Lactobacillus rhamnosus* R11, *Lactobacillus plantarum* 299V, *Bifidobacterium animalis* Bo and *Bifidobacterium animalis* Bb12, and also, on pathogenic strains such as *Listeria monocytogenes* ESB 3562, *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076 and *Bacillus cereus* NCTC 2599. This study will allow advancing with a screening on the effect of this compound in the gut microbiota.

## 2. MATERIALS AND METHODS

### 2.1 Antimicrobial screening

#### 2.1.1. Sample preparation ($SO_2$)

The salt of sodium sulphate ($Na_2SO_4$ 99.0%) anhydrous (CARLO ERBA REAGENTS, France) was dissolved in sterilized de-ionized water to obtain sulphur dioxide ($SO_2$) with concentration of 500 mg/L and 1000 mg/L.

#### 2.1.2. Microorganisms and culture medium conditions

Various probiotic and pathogenic bacterial strains were used to investigate the effect of sulphur dioxide ($SO_2$) on them. The health promoting probiotic strains were *Lactobacillus acidophilus* KI, *Lactobacillus rhamnosus* R11, *Lactobacillus plantarum* 299V, *Bifidobacterium animalis* Bo and *Bifidobacterium animalis* Bb12. The pathogenic bacterial strains were *Listeria monocytogenes* ESB 3562 (*CBQF-Centro de Biotecnologia e Quimica Fina-Laboratorio collection)*, *Escherichia coli* ATCC 25922, *Salmonella enteritides* ATCC 13076, and *Bacillus cereus* NCTC 2599. All probiotic strains were grown in MRS broth medium aerobically at temperature 37 $^0$C for 24 h to pre-cultured them except *B. animalis* Bo and *B. animalis* Bb12, which were grown in MRS broth medium anaerobically supplemented with L-cystein-HCL 0.5 g/L (0.05%) to scavenge oxygen. Pathogenic strains were grown in Mueller Hinton broth aerobically at 37 $^0$C for 24 hr to pre-cultured them.

#### 2.1.3. Growth curves of probiotic bacterial strains with $SO_2$

The test solutions of $SO_2$ were prepared with $Na_2SO_4$ anhydrous dissolves in de Man, Rogosa and Sharp (MRS) broth (Biokar Diagnostics, Beauvais, France) to reach the concentration of 1000 mg/L and 500 mg/L and then filter sterilized by passing through 0.22 µm pore filter (Frilabo, Maia, Portugal). Then, these test solutions were inoculated with probiotic bacterial strains at 1% (V/V), using an inoculum of ca. $10^8$ CFU/mL. Then growth mixture was transferred with micropipette by pouring of 200 µl volume in to each well of the micro plate having 96 wells. The plate was incubated at 37$^o$C for 24 hr and was monitored by measuring with optical density at wavelength of 660 nm. A microplate reader was used for their incubation. Positive control was made by bacterial inoculation of MRS broth only, while keeping negative control by having only MRS broth without inoculation. The test solution was filtered through 0.22 µm filter (Frilabo, Maia, Portugal) for their sterilization and to prevent degradation of ($SO_2$) in the media. All the test solutions of individual bacterial strain were manipulated in triplicate. MRS broth for *B. animalis* Bo *and B. animalis* Bb12, was supplemented with filter-sterilized 0.5g/L (0.05%) of L-cysteine-HCL (Fluka, Switzerland), and wells of micro plate were covered with 50 µl of autoclaved-sterilized liquid paraffin (Merck, Germany), to avoid the presence of oxygen (Sousa *et al*., 2015, Silva, 2012).

The growth curve of *B. animalis* Bo with $SO_2$ 1000 mg/L was also studied regarding their viable cell counts. For that, the inoculum was pre-cultured within MRS broth having filtered-sterilized 0.5 g/L (0.05%) of L-Cysteine (Fluka, Switzerland) and was incubated anaerobically at 37$^o$C for 24 hr. The test solution $SO_2$ 1000 mg/L was prepared in 50 mL Falcon tube with MRS broth with 0.5 g/L of L-cysteine in deionized water, which was filtered through a sterile filter having pore size of 0.22 µm. It was inoculated at 10% (V/V) with an inoculum of ca. $10^8$ CFU/mL from pre-cultured MRS, incubated anaerobically at 37$^o$C for 24 hr. Samples were collected at 0, 2, 4, 6, 8, 12 and 24 hr and decimal dilutions were performed with ($10^{-1}$-$10^{-6}$) in sterilized peptone (Sigma-Aldrich) water. For plating of MRS

agar (L-cysteine), drop method technique was used as described by Miles *et al.,* (1938). A positive control of inoculated MRS broth was used without being poured the test compound ($SO_2$). All the plates were incubated for 48 hr at 37$^oC$ anaerobically. Colonies were counted and results were presented by plotting the log CFU versus time. All assays were performed in duplicate. Whenever a result was below the quantification limit, the methods detection limit (log 50) was assumed.

**2.1.4. Growth curves of pathogenic bacterial strains with $SO_2$**

The same procedure was followed as described above, where the test solutions of $SO_2$ were prepared using $Na_2SO_4$ salt (anhydrous) having concentration of 1000 mg/L and 500 mg/L by dissolving in Mueller-Hinton broth (MHB) (Biokar Diagnostics, Beauvais, France) and then are filter sterilized by passing through filter having pore of 0.22 µm. And then, these solutions were inoculated with pathogenic bacterial strains 1% (V/V) inoculum of ca. $10^8$ CFU/mL. Then, growth mixture was transferred with micropipette by pouring 200 µl volume in to each well of the micro plate having 96 wells. The plate was incubated at 37$^oC$ for 24 hr and was monitored by measuring with optical density at wavelength of 660 nm. A microplate reader was used for its incubation. Positive control was made by inoculation of MHB broth only, while keeping negative control by having MHB broth without inoculation. The test solutions were filtered through 0.22 µm filter (Frilabo, Maia, Portugal) for their sterilization to prevent degradation of test compound ($SO_2$) in the media. All the test solutions having individual bacterial strain was manipulated in triplicate.

**2.2. Quantification of $SO_2$ in gastrointestinal tract (GIT) simulation model**

The interaction of $SO_2$ with other molecules from GIT such as gastric enzyme, (pepsin) and intestinal enzyme (Pancreatin) with bile salts using an in vitro GIT simulation model was monitored. Furthermore, it was also observed that $SO_2$ had been changed into different interconvertible chemical forms, such as bisulphites ($HSO_3^-$) and sulphites ($SO_3^{2-}$), which passes through mouth, stomach and intestines in GIT model. The pH being acidic and basic in gastric and intestinal environment was also maintained and their effect upon $SO_2$ was noticed in GIT simulation model.

**2.2.1 Sample and simulated GIT model preparation**

In order to simulate gastrointestinal tract model to quantify $SO_2$, we have used the conditions described by Madureira *et al.* (2011). Samples of $SO_2$ were prepared by dissolving $Na_2SO_4$ anhydrous (Purity 99%) in ultra-pure water with three different concentrations 200 mg/L, 400 mg/L and 800 mg/L. Solutions were filtered through 0.22 µl micropore filter (Frilabo, Maia, Portugal) for their sterilization. All these solutions were exposed to simulated conditions of mouth, stomach and small intestine, having all the enzyme juices present in each stage except amylase, which was eliminated because of reduced time to interact with the liquid test solutions, but still basicity of the mouth condition was maintained. The simulated pH, temperature, and peristaltic movement were also maintained before exposition of $SO_2$ to GIT model. At each stage of mouth, stomach and intestine, samples were collected in duplicate, one for $SO_2$ quantification by HPLC and another for determination of charged particles (zeta potential, ZP) and particle size (PS) using dynamic light scattering (DLS).

**2.2.2 Mouth simulation condition**

The pH was calibrated at 6.9 using 1 M of $NaHCO_3$ and the saliva human amylase) was not incorporated, since the contact was very short (2 min) not inducing any change in the $SO_2$.

### 2.2.3 Stomach simulation condition

The pH was calibrated at 2.5 using 1 M HCL and gastric juice was simulated by dissolving pepsin (Sigma-Aldrich) 25 mg/mL in 0.1 N =0.1 M of HCL, which was added at the rate of 0.005 mL/mL of sample. Incubation was done at 37$^{o}$C for 1 hr and 130 rpm.

### 2.2.4 Small intestine simulation condition

Intestinal juice was prepared by dissolving 2 g/L of pancreatin (Sigma-Aldrich) and 12 g/L of bile salts (Sigma-Aldrich) in 0.1 M of $NaHCO_3$. After, it was poured at the rate of 0.25 mL/mL in to test solutions ($SO_2$) of the simulated model. Consistently, pH was also adjusted to 6.5 using 1 M $NaHCO_3$. All the test solutions were incubated for whole duration of 90 min, at 37$^{o}$C and 45 rpm. All assays were performed in duplicate. Each sample was taken out with 2 mL (1 mL for HPLC and 1 mL for DLS) after each 30 min interval of time and were collected in ice chilled Eppendorf tubes on ice bath. And then, all these samples were transferred to Medical freezer (SANYO) and stored at -80$^{o}$C, to preserve $SO_2$ and not to have any chemical reaction.

All the enzyme solutions were prepared freshly and filter-sterilized using a 0.22 µm-membrane filter (Frilabo, Maia, Portugal) prior to use. After sterilization, all the solutions were maintained in an ice bath during the entire period of simulation prior to gradual addition into GIT simulation model. To simulate human body temperature, the water bath was maintained at 37$^{o}$C. while, the mechanical agitation was used to parallel peristaltic movements, with emphasizing the intensities resembling those attained in each digestive compartment.

### 2.2.5 Quantification of $SO_2$

Quantification of $SO_2$ was performed by high performance liquid chromatography (HPLC) described by Mcfeeters and Barish (2003) using UV-IR detectors (K-250-1 and K-230-1, KNAUER). A separation column BIO-RAD, Aminex® HPX-87H Ion Exclusion Column (300mm x 7.8mm) was used. Chromatographic separation was carried out with mobile phase of solvent of the diluted sulphuric acid solution of 5 mM. The column was eluted isocratically and for that purpose, an HPLC PUMP K-1001 (WellChrom, KNAUER) at a flow rate of 0.6 mL/min was used. The column temperature was maintained at 41 $^{o}$C in an auto-sampler column oven (Eldex CH-150). Injection volume of 20 µl was used to inject the sample on column. The peaks of the spectra were obtained for $SO_2$ at 210 nm. It was analysed by comparison of retention time and spectra with that of several pure standards of $SO_2$ having concentrations of 10, 20, 50, 100, 200, 500, 1000 (mg/L), which were prepared in (0.01M) of sulphuric acid ($H_2SO_4$). Analysis was performed in duplicate.

## 2.3. Inactivation of pathogenic and probiotic bacterial strains with sulphur dioxide ($SO_2$) in gastrointestinal tract simulation model

To see the effect of sulphur dioxide ($SO_2$) upon probiotic and pathogenic bacterial strains, bacterial cells were also exposed to gastric (pepsin) and intestinal (bile and pancreatin) juices along with $SO_2$.The pH value of the gastric 2.0-2.5 and intestine 6.5 were also maintained in the simulated model.

### 2.3.1. Pathogenic bacterial strains inactivation in simulated gastric model

All the probiotic and pathogenic bacterial strains growth culture was maintained with their viable cells number of approximately $10^8$ CFU/mL for the experimental purposes. Bacterial cells were grown with their normal growth rate having viable cells number of ca.$10^9$ CFU/mL . Therefore, 1-fold dilution was

made for all the bacterial strains to maintain the desirable viable cells number ca. $10^8$ CFU/mL by having 1 log reduction before the experiment.

The pathogenic bacterial strains were subculture with 10% (V/V) in Mueller-Hinton (MH) Broth (Biokar Diagnostics, Beauvais, France), using a pre-inoculated cultured media, with the total volume of 20 mL in each falcon tube and were incubated for overnight of 18 hr at 37$^o$C aerobically. Those cultured falcon tubes were centrifuged at 4000 rpm, at 4$^o$C for 10 min in automatic thermo-stabilizing centrifuge (Universal 32R, Hettich, Germany). After centrifugation, the supernatant was discarded and the pellet was resuspended in 1 g/L (0.1%) of sterilized peptone water (SIGMA, Germany) with sodium chloride (NaCl 0.85%) using the same volume of supernatant already discarded. Then 10% of inoculum of the resuspended cells were transferred to two more tubes having replica: control 01 was sterilized peptone water 1 g/L (0.1%) with NaCl (0.85%) , and second tube contained compound solution of $SO_2$ (1000 mg/L) dissolved in sterilized peptone water 1g/L (0.1%) with NaCl (0.85%) having viable cells number at ca. $10^8$ CFU/mL . In accordance to gastric simulation, pH was adjusted to 2.0-2.5 using 1 M HCL, and gastric juice, pepsin (Sigma-Aldrich) with 25 mg/mL was prepared in 0.1 M HCL, which was added at the flow rate of 0.005 mL/mL to the test solutions having viable cells and those controls. All the falcon tubes were incubated for 240 min at 37$^o$C aerobically. During incubation time, samples were collected at 0, 30, 60, 90, 120 and 240 min. Serial dilution was made in peptone water and then plated by Miles and Misra methods in Mueller-Hinton Agar except for *Bacillus Cereus* NCTC2599, which was plated on Plate Count Agar (PCA) because of the spreading of the colonies. The plates were incubated aerobically at 37$^o$C for 24 hr. The colonies were counted, and the viable cell numbers were reported as CFU/mL.

**2.3.2 Probiotic bacterial strains inactivation in simulated Intestinal model**

The probiotic bacterial strains were subculture with 10% (V/V) in MRS broth (Biokar Diagnostics, Beauvais, France) using a pre-inoculated cultured media with 20 mL in falcon tube and were incubated for 18 hr at 37$^o$C aerobically, except for two strains of probiotic, *B. animalis* Bo and *B. animalis* Bb12, which were incubated anaerobically at 37$^o$C for 18 hr. Those cultured falcon tubes were centrifuged at 4000 rpm, at 4 $^0$C for 10 min in an automatic thermo-stabilizing centrifuge (Universal 32R; Hettich, Germany). After centrifugation, the supernatant was discarded and the pellet, which is content of viable cells, were resuspended with sterilized peptone (SIGMA, Germany) water of 1 g/L (0.1%), with sodium chloride (NaCl 0.85%), and using the same volume of supernatant already discarded. Then 10% of inoculum of the resuspended cells were transferred to three more tubes having replica: control 01 was just sterilized peptone water 1 g/L (0.1%), Control 02 was sterilized peptone water 1 g/L (0.1%) with NaCl (0.85%) , and third tube contained compound solution of $SO_2$ (1000 mg/L) dissolved in sterilized peptone water 1g/L (0.1%) with NaCl (0.85%) having viable cells number at ca. $10^8$ CFU/mL. In accordance to intestinal simulation, pH was adjusted to 6.0-6.5, using 1 M $NaHCO_3$, and intestinal juices, pancreatin (Sigma-Aldrich) with 2 g/L and bile salts (Sigma-Aldrich) with 12 g/L was prepared in 0.1 M $NaHCO_3$, which were added at the flow rate of 0.25 mL/mL to the test solutions having viable cells and controls. All the falcon tubes were incubated for 24 hr at 37$^o$C aerobically except of two strains, *B. animalis* Bo and *B. animalis* Bb12, which were incubated anaerobically. During incubation time, samples were collected at 0, 2, 4, 6, and 24 hr. Serial dilution was made in peptone water and then plated by Miles and Mirsa methods in MRS Agar. And plates were incubated aerobically at 37$^o$C for 48

hr except *B. animalis* Bo and *B. animalis* Bb12, which were incubated anaerobically. The colonies were counted, and the viable cell number were reported as CFU/mL. All the experimental samples were manipulated in duplicates.

**2.4. Zeta potential and Zeta size of $SO_2$**

Medium diameter of $SO_2$, enzyme-$SO_2$ complex in the gastric system (pepsin) and in intestinal system (bile, pancreatin), and as well, relative zeta potential was assessed using a Zeta Sizer, Nano ZSP (Malvern, UK). Dynamic light scattering (DLS) was also used to assess complexes diameter with the intensity distribution weighted according to the scattering intensity of each particle fraction or family. Zeta potential was measured using Laser Doppler Anemometry (LDA). All analyses were carried out with an angle of $90^o$ at $25^oC$.

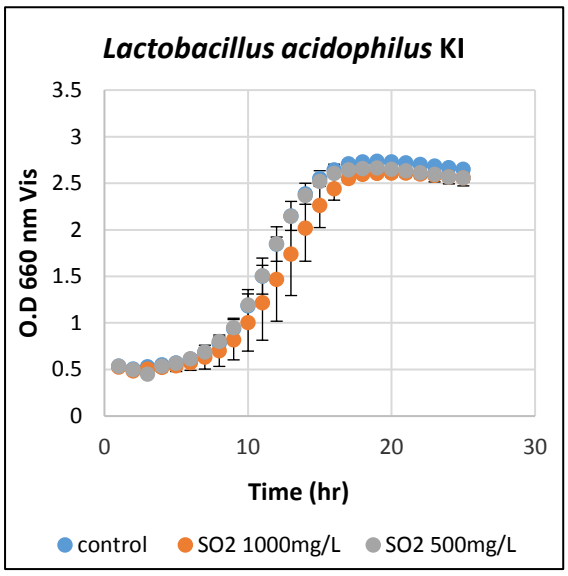
**2.5. Metabolism of *Bifidobacterium animalis* Bo with $SO_2$**

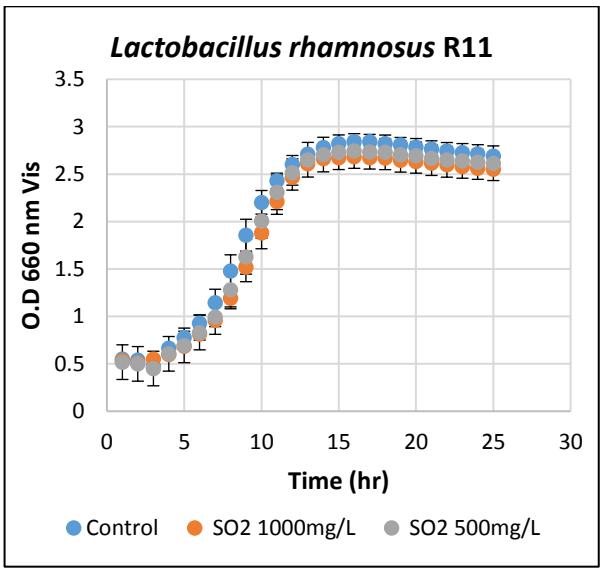
The metabolism of *B.animalis* Bo with $SO_2$ was studied to determine the effect on organic acids production, namely citric acid, acetic acid, lactic acid, succinic acid, propionic acid and butyric acid. Additionally, the effect of $SO_2$ on glucose consumption was also performed by high-performance liquid chromatography (HPLC). Optical density was measured at 210 nm, using an ultra violet spectrophotometer (UV Detector K-2501, KNAUER) for the analysis of propionic and butyric acids production, while, infra-red spectrophotometer (RI Detector K-2301, KNAUER) was used for the analysis of glucose consumption and citric, acetic, lactic and succinic acids production. The column HPX-87H (Aminex® 300mm x 7.8mm, ion Exclusion Column) with the flow rate of 0.8 mL/min was maintained. The temperature of the column was maintained at $41^oC$. The column was diluted isocratically with the diluted solution of 5 mM of sulphuric acid solution (slight modification of method from McFeeters and Barish, 2003). Standards of organic acids and sugars were used to perform the identification and quantification: glucose in the range (0.8-20 g/mL), lactic acid (0.07-22 g/L), citric acid (0.08-5 g/L), succinic acid (0.08-5 g/L), acetic acid (0.25-25 g/L), propionic acid (0.008-2.5 g/L), butyric acid (0.009-2.4 g/L).

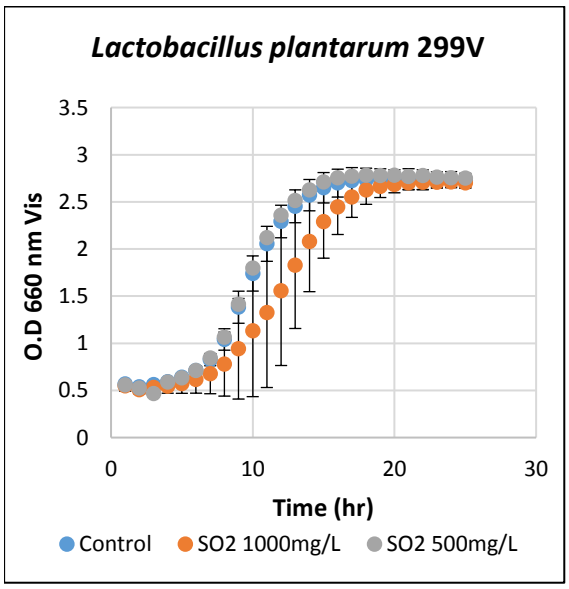
## 3. Results and Discussions

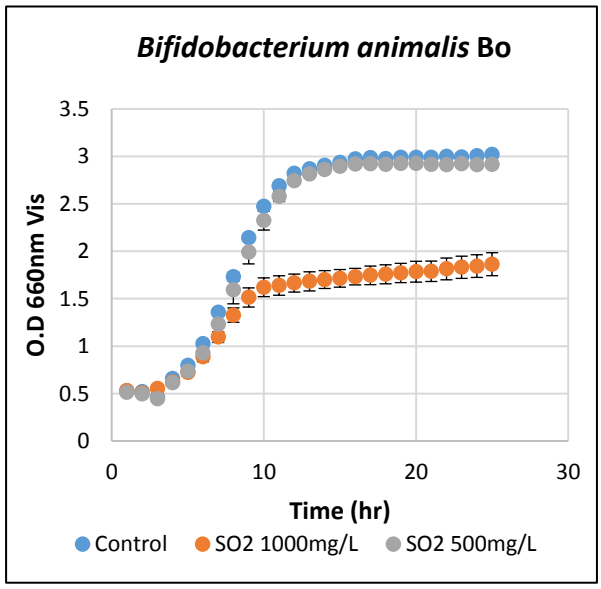
### 3.1.1 Growth curves of probiotic bacterial strains with $SO_2$

The growth curves of probiotic bacterial strains with $SO_2$ were investigated and results were obtained by measuring optical density of 660 nm (visible light) for 24 hr in a microplate reader. Figure 3.1 displays the growth curves of probiotic bacteria growing at two concentrations of $SO_2$ (500 mg/L and 1000 mg/L). The results showed that $SO_2$ at 500 mg/L had no negative effect on growth of the probiotic bacterial strains. Concerning the concentration of 1000 mg/L, all the bacteria were slightly inhibited, except for *L. rhamnosus* that was not affected. In the case of *B. animalis* Bo the viable cell numbers decreased ca. 50% compared to the control. The effect on the other strains resulted mainly in a delay on the entrance on the exponential phase for *L. plantarum.* Hence, the susceptibility of probiotic bacteria to $SO_2$ only occurs at very high concentrations (1000mg/L), with an intensity in decreasing order *Bifidobacterium animalis* Bo > *Bifidobacterium animalis*> BB12, *L. plantarum* > *L. acidophilus* > *L. rhamnosus*.



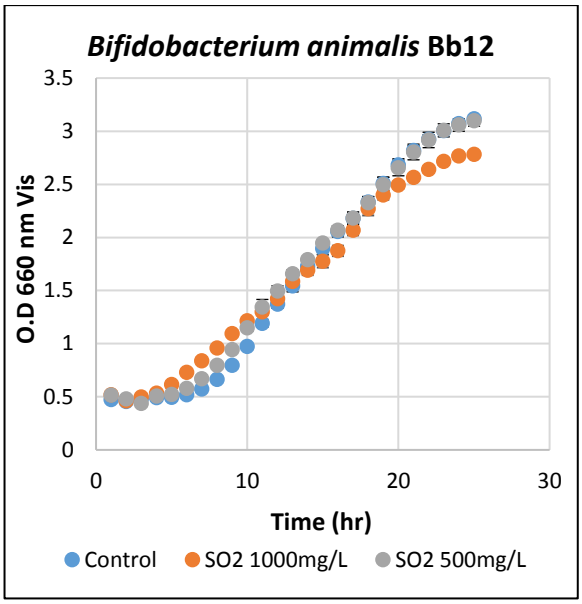



**Figure 3.1 -** Growth curves of all the probiotic strains with sulphur dioxide ($SO_2$) 500 mg/L and 1000 mg/L. All test assays were performed in triplicate.

Since the *B. animalis* Bo was the most affected by $SO_2$, and the optical densities does not allow us to conclude about viability since it measures all cells viable and non-viable, a growth curve of *B. animalis* Bo with $SO_2$ 1000 mg/L and the enumeration of viable cell numbers that grow in media plate was performed. $SO_2$ had not any prominent effect upon this bacterial strain as shown in figure 3.2, which displays the growth curve of *B. animalis* Bo grown with $SO_2$ 1000 mg/L compared to a control without $SO_2$. *B. animalis* Bo without $SO_2$ showed a curve with normal growth rate reaching ca. 5 x $10^9$ CFU/mL of maximum growth in the stationary phase. When cultured with $SO_2$, the growth rate was affected and stationary phase started at 12 hr instead of 8 hr and the maximum growth in the stationary phase was reduced ca. 1 log cycle (ca. 5 x $10^8$ CFU/mL) comparing with control.

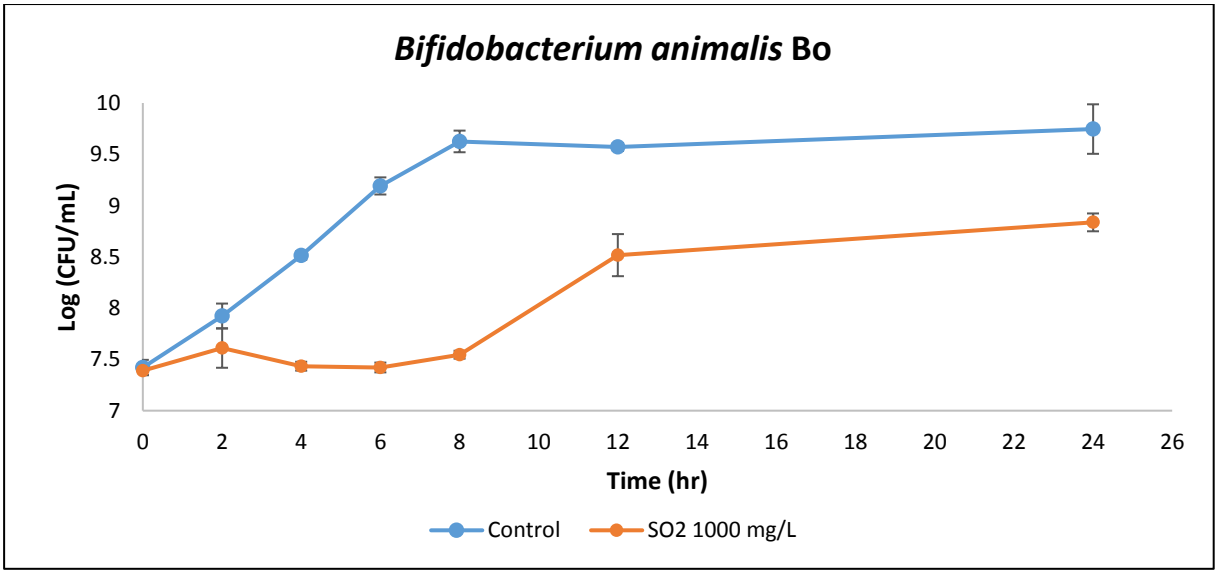



**Figure 3.2 -** Growth curve of *Bifidobacterium animalis* Bo with sulphur dioxide ($SO_2$) 1000 mg/L in enumeration of viable cells. All assays were performed in duplicate.

### 3.1.2 Growth curves of pathogenic bacterial strains with $SO_2$

The growth curves of pathogenic bacterial strains with $SO_2$ after incubation for 24 hr were obtained using optical densities measurements in a microplate reader and presented in Figure 3.3. In the case of *L. monocytogenes,* the lower concentration showed no inhibitory effect, but the higher concentration (1000 mg/L) showed an inhibition of growth with a reduction of maximum growth of ca. 40%. On *E. coli* a similar effect was obtained with a reduction observed only at the highest concentration (1000 mg/L). In the case of *Salmonella* and *B. cereus*, the $SO_2$ at concentrations of 500 mg/L or 1000 mg/L had a negative effect on the growth, although in the case of *Salmonella* a delay on the beginning of the exponential phase was observed for both concentrations.

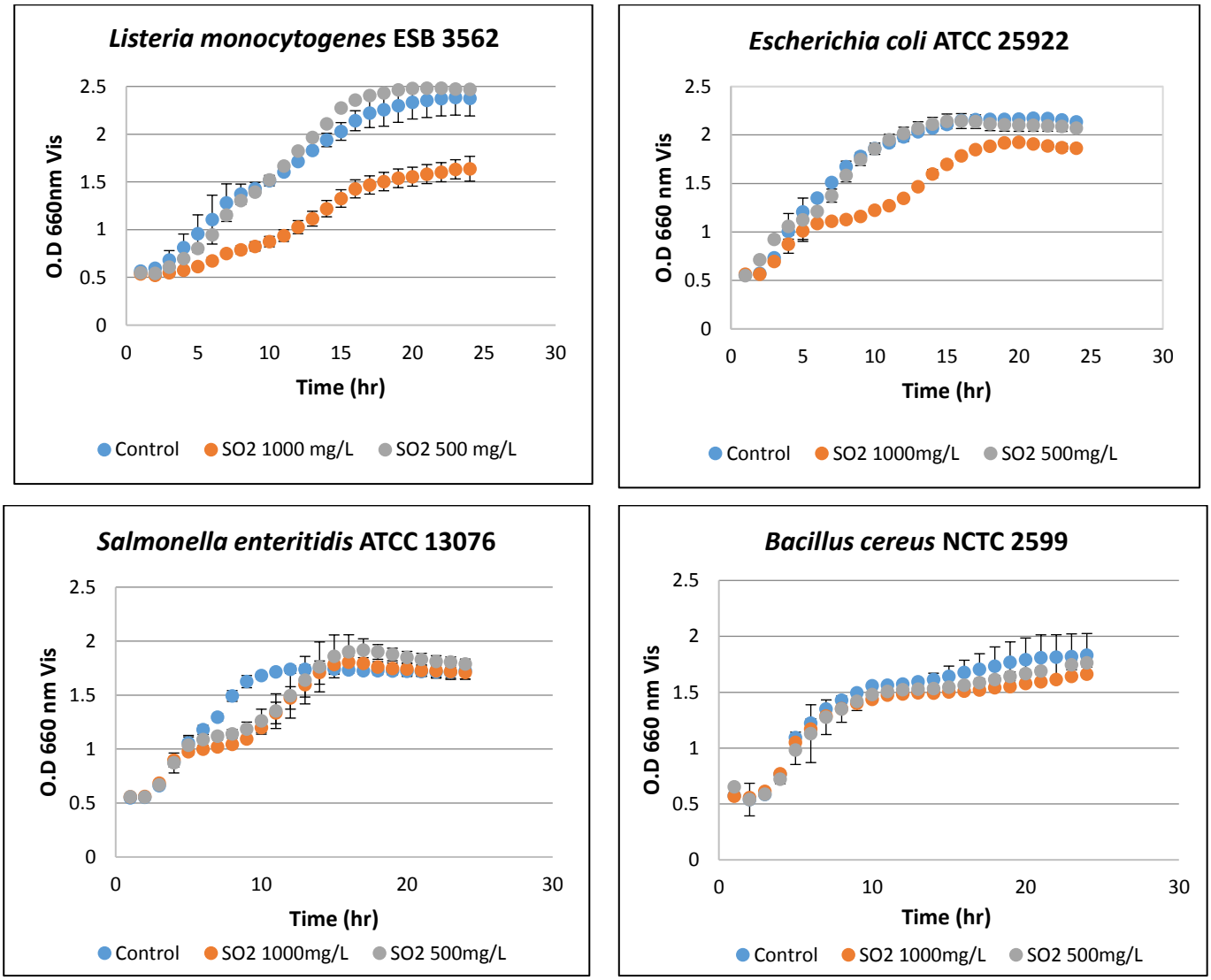



**Figure 3.3 -** Growth curves for all the pathogenic bacterial strains with sulphur dioxide ($SO_2$) 500 mg/L, 1000 mg/L. All test assays were performed in triplicate.

### 3.2.1 Inactivation of pathogenic bacterial strains in gastric simulated model system

Bacterial cells were suspended in peptone water + NaCl (0.85%) to create the isotonic environment to bacterial cell membrane having balanced NaCl ($Na^+$ and $Cl^-$) charged ions inside and outside the cell membrane. Isotonic solution of NaCl (0.85%) is used as a "non-nutrient" solution, for comparison with bacterial killing rates in growth media, since deionized water provoke cell lysis by osmotic shock (Engelkirk *et al.,* 2011, Londono *et al.,* 2017).

The effect of $SO_2$ 1000 mg/L upon *L. monocytogenes* ESB 3532 in simulated gastric conditions is presented in Figure 3.4. As can be seen, $SO_2$ didn't have any effect since there was no reduction in viable cells count during incubation time of 240 min. This result is similar to the result obtained by Koseki *et al.* (2010), in which *L. monocytogenes* there was not significant reduction in numbers of bacteria (< 0.5 $log_{10}$ CFU/mL at pH 2.2) when exposed to similar conditions. Control shown a reduction in their viable cells counts (ca. 5 logs) until 120 min interval of incubation time, but until 240 min, with $SO_2$ at 1000 mg/L protective effect is shown when this bacteria is under gastric conditions. This phenomena must be further investigated. Pepsin is known to have a antimicrobial effect in bacteria (Zhu *et al.* 2006), so maybe $SO_2$ inactivated somehow pepsin and so the bacterial cells were able to survive to these conditions.

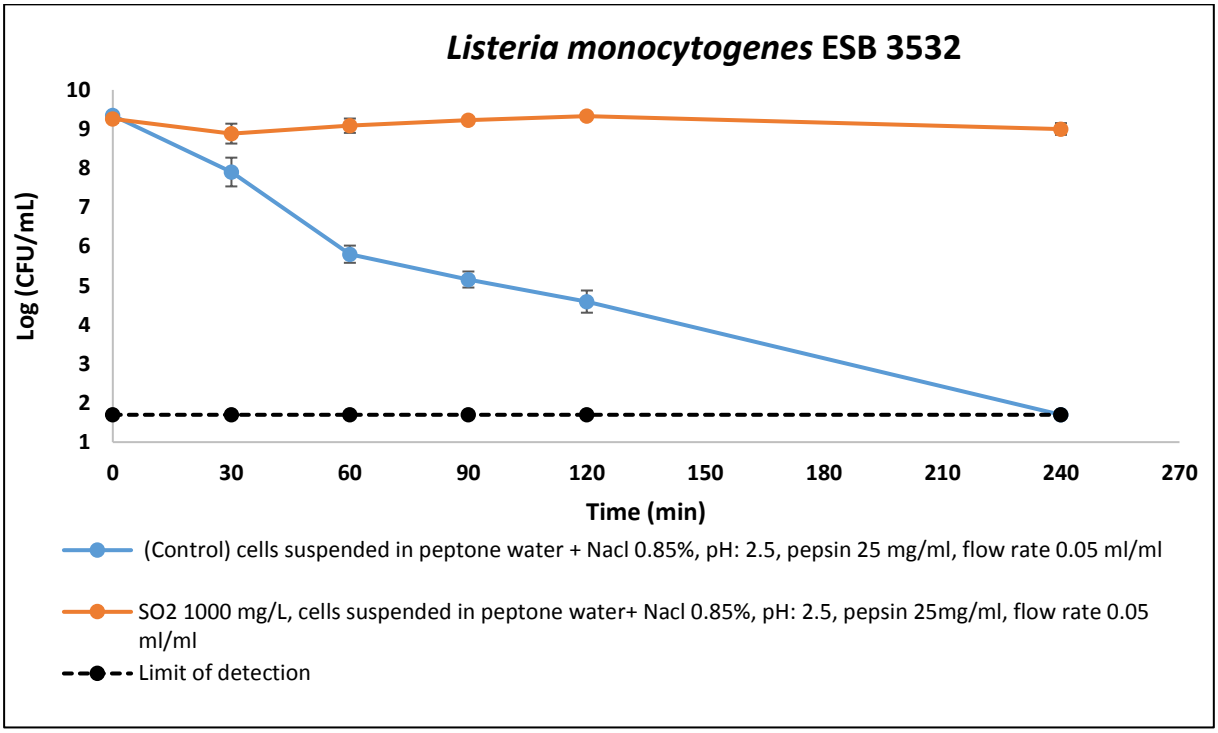



**Figure 3.4 -** Viable cell numbers of *Listeria monocytogenes* ESB 3532 when exposed to the simulated gastrointestinal conditions in the presence of $SO_2$ (1000 mg/L) or absence (control).

The effect of $SO_2$ 1000 mg/L upon *Escherichia coli* ATCC 25922 in simulated gastric conditions is presented in figure 3.5. The $SO_2$ didn't show a negative impact upon viable cells of *E. coli* throughout entire incubation time for 240 min. In the case of control without $SO_2$ 1000 mg/L the contact of *E. coli* with pepsin and pH 2.5 showed a slight reduction (2 log cycles) after 90 min of contact. On average

around 20-80% of ingested *E. coli* are estimated to arrive in the small intestine without inactivation by low pH (Takumi *et al.,* 2000). While, Koseki *et al.* (2010) and Takumi *et al.* (2000) showed that in a gastric simulation, the pathogenic strain *E.coli O157:H7* didn't experience any prominent effect of reduction and inactivation when exposed to gastric juices with low pH (2.5). In addition to support their results, by consumption of food, the pH of the gastric system (stomach) not remain constantly acidic, but it may temporarily increase. And, pathogenic bacteria can be transported to small intestine and the chances of survival could be high. Actually, the same authors say that *E. coli* in healthy young adults, 20-73% of the ingested bacteria could survive and arrive to the small intestine.

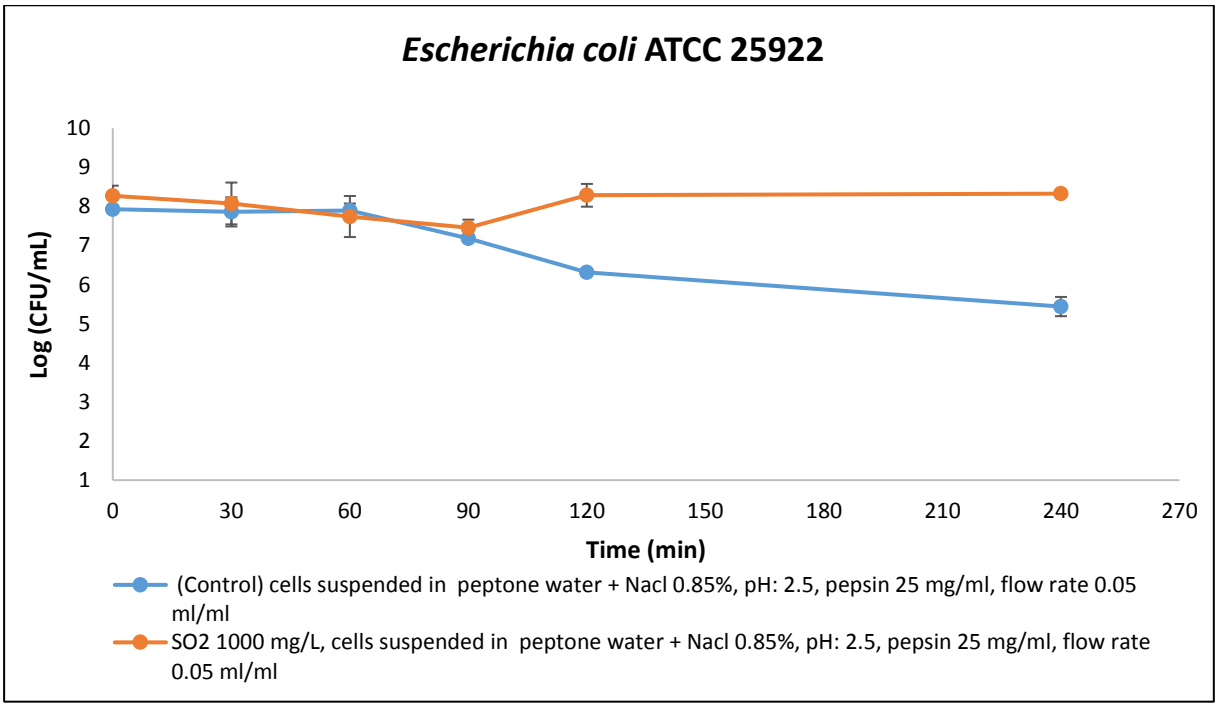



**Figure 3.5 -** Viable cell numbers of *Escherichia coli* ATCC 25922 when exposed to the simulated gastrointestinal conditions in the presence of $SO_2$ (1000 mg/L) or absence (control).

The effect of $SO_2$ at 1000 mg/L upon *Salmonella enteritidis* ATCC 13076 in simulated gastric conditions is presented in figure 3.6. The results showed that for this food pathogen the $SO_2$ at 1000 mg/L did not inhibit *S. enteritidis* under gastric conditions, since no decrease was observed along the 240 min of incubation. However, the positive control (without $SO_2$) abruptly lost their viability with specific interval of time up to 90 min, and afterwards, there were no viable cells until the end of incubation. According to Silva *et al.* (2016), *S. enteritidis* CCS3 inoculated in pork meat suffered a reduction on viable cells of ca. 1.4 log cycles after exposed to simulated gastric fluid at pH 1.5 for up to 3 hr. Based in these results we may hypothesise that the $SO_2$ may protect the bacteria under gastric conditions.

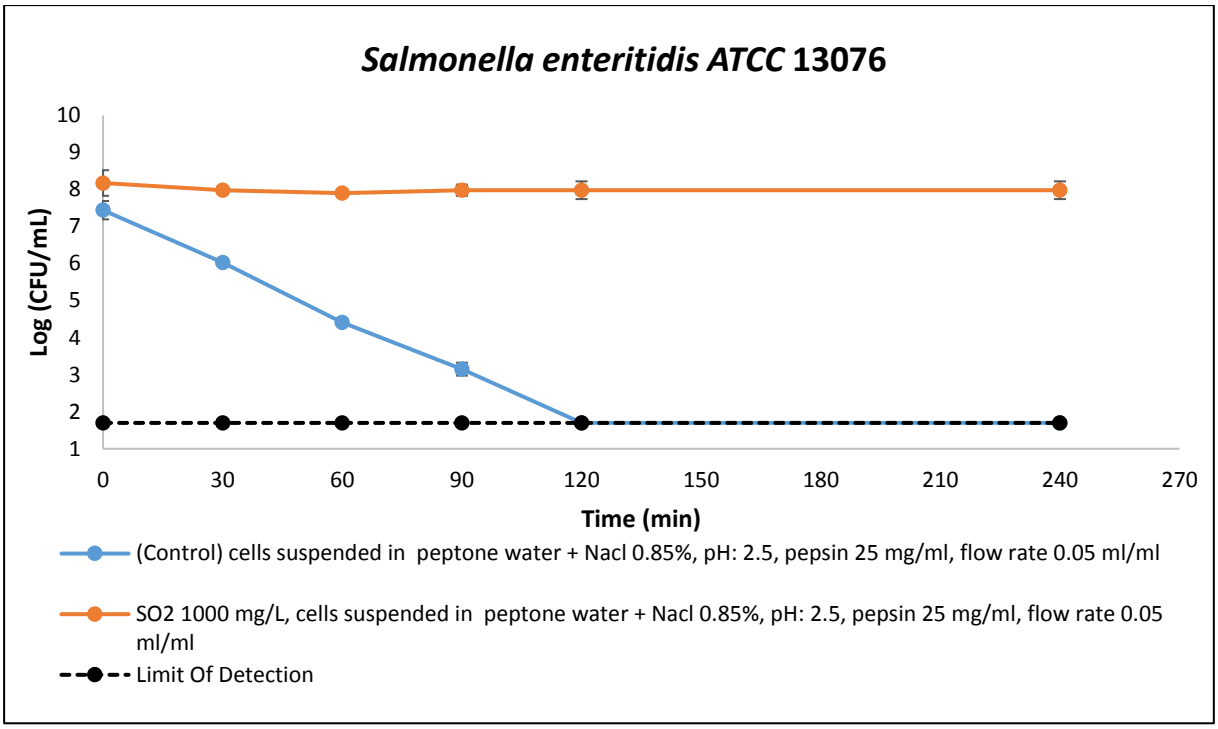



**Figure 3.6 -** Viable cell numbers of *Salmonella enteritidis* ATCC 13076 when exposed to the simulated gastrointestinal conditions in the presence of $SO_2$ (1000 mg/L) or absence (control).

The effect of $SO_2$ at 1000 mg/L upon *Bacillus cereus* NCTC 2599 in simulated gastric conditions is presented in figure 3.7. Once again, $SO_2$ didn´t have any negative effect on bacterial cells viability. Nevertheless, even without $SO_2$ there was no negative impact on viable cells number. According to a previous work by Ceuppens *et al.* (2012) on inactivation of *B. cereus* contaminated lasagne verde with 7.0 to 8.0 log CFU/mL vegetative cells subject to *in vitro* simulation of gastrointestinal conditions, after 30 min of time in the stomach pH 5.0-4.0, *B. cereus* cell numbers lowered ca.1 log. But, when the pH of the gastric decreased below 4.0, the vegetative cells were rapidly inactivated, in contrast for the inactivation of *B. cereus* with $SO_2$ 1000 mg/L in gastric system, in which, the bacterial strain survived to the adverse and unfavourable conditions. Ceuppens *et al.* (2012) performed work on the inactivation of *B. cereus* contaminated mashed potatoes and exposed to gastrointestinal tract conditions on batch and dynamic incubations. In batch incubations, there was a rapid inactivation in TSB (Tryptone Soy Broth) with a pH lower and equal to 4.5, and as well as gastric medium with a pH lower and equal to 4.0. The reductions were more or equal to 3 logs within around 1 hr. Whereas, in dynamic experiments, the slow pH change along with the addition of gastric medium to the mashed potato medium containing *B. cereus,* resulted in a high survival of the vegetative cells at a pH greater or equal to 4.5 and were inactivated at a pH value lower or equal to 4.0 resulting in complete die-off after 2 hours in the stomach simulation. Wijnands *et al.* (2009), investigated the survival of *B. cereus* in a gastrointestinal simulation model using a concentration of $10^5$ CFU/mL and also followed the stationary and exponential phase bacterial cells inactivation. The model mimicked the real gastric condition of the stabilization of gastric pH after the consumption of meal of solid food. Also the simulation of the gastric conditions in young and elderly health adults with the consumption of a solid meal. These works revealed that the survival chances of

the vegetative cells of *B. cereus* are putatively higher in healthy young adults than young, and there was only 1 to 2 log-units reduction for both exponential and stationary phase vegetative cells. The inactivation didn't start at maximum pH, but, at 4.5, for the exponential phase and at 4.0 at the stationary phase, which doesn't agree with the present experimental work results in which most of the bacterial strain survived the gastric conditions, even with much lower pH 2.5 and as well as with antimicrobial compound of $SO_2$ (1000 mg/L).

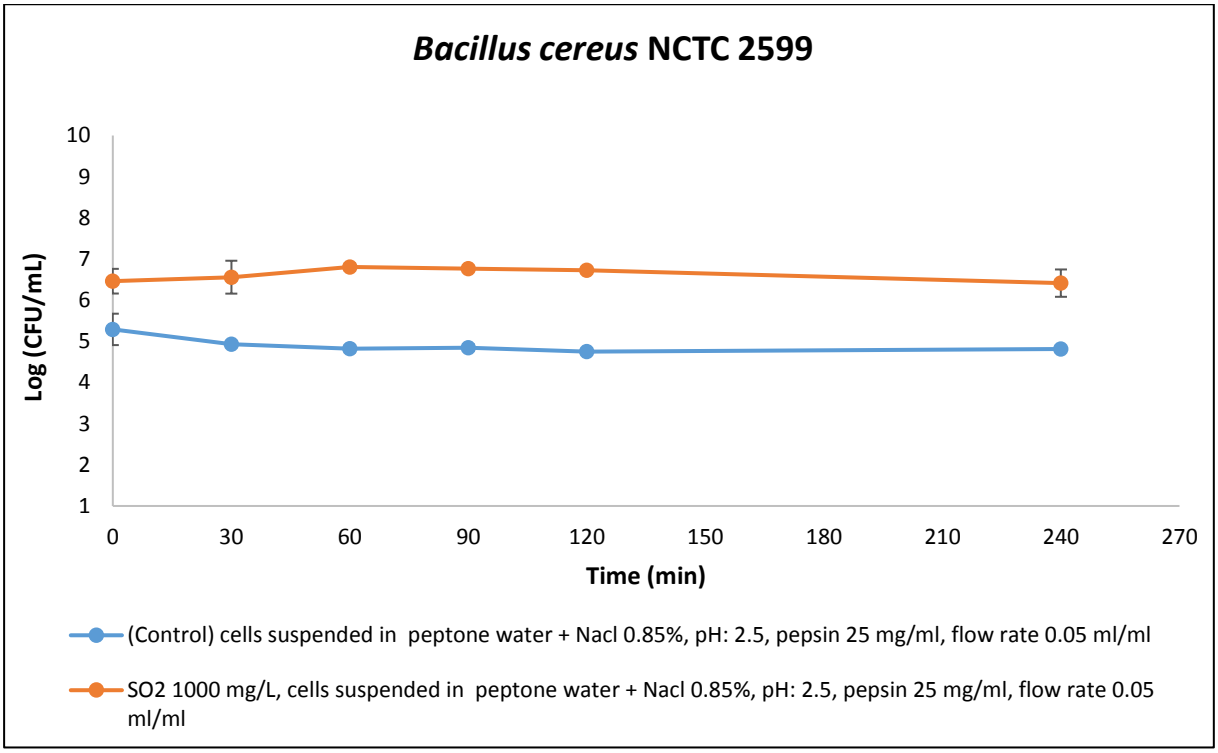



**Figure 3.7 -** Viable cell numbers of *Bacillus cereus* NCTC 2599 when exposed to the simulated gastrointestinal conditions in the presence of $SO_2$ (1000 mg/L) or absence (control).

According to Koseki *et al.* (2010), the amount of pathogen reduction had no correspondence with actual stomach digestion, in a model developed for inactivation of the pathogenic microorganisms in simulated digestion process for each food. Because, various types of foods in large quantity are ingested together in an actual meal, the pH in the stomach will not decrease to a sufficiently low pH that is required for inactivation. Furthermore, approximately 50% of the meal would be transported to the intestine within 120 min in an actual digestion process. So, before the passage of food to the intestine, the mixture of food and gastric juice would not lower the pH level to inactivate the pathogenic bacteria. The survival of *L. monocytogenes* and *E. coli* O157:H7 has remarkable resistance towards acidic environment. But the actual cause of resistance towards pathogenic microorganisms is due to the pH increase with the ingestion of food which gives more chance to survival. Silva *et al.* (2016) further emphasized that, bacteria exposed to mild acid stress leads towards resistance and may survive in similar and different stress conditions due to a cross protection effect. Moreover, moderate acidic conditions could also trigger resistance to gastric fluid in *S. enterica,* increasing the risk and severity of illness.

Leyer and Johnson (1993), mentioned that, *Salmonella typhimurium* can show resistance towards environmental stresses due to adaptation by inducing specific set of genes termed stimulons, which shows adaptive response to acid, salt, heat, $H_2O_2$ and oxygen radicals. Such adaptation plays critical role in invoking tolerance enhancement towards these stresses and may promote survival efficiency in such adverse environment.

Hence, the food acidity is an imperative issue for the bacterial cell's surveillance, because the mild acid of food or drink induces tolerance in vegetative cells prior expose to acid, and plays an important role in the endurance towards gastric fluid. This results in lowering down the rate of inactivation of vegetative cells and in even high numbers of surviving cells of *B. cereus*. In addition to stomach pH, gastric emptying also plays an important role, in which the food type influences this phenomena and is reflected by the lag time between gastric filling and gastric emptying. Liquids pass through almost immediately after entering the stomach while, solid foods experience a lag period and left behind, and *B. cereus* gastric passage may also have affected. Moreover, some of individual characteristics such as neural, hormonal and intestinal feedback stimuli are important determinants which may influence the gastric environment (Wijnands *et al.,* 2009).

**3.2.2 Inactivation of probiotic bacterial strains in intestinal simulation model**

The incorporation of salt NaCl (0.85%) was to keep the bacterial viable cells in isotonic condition and not cell lyse because of hypertonic condition. As the effect was almost the same for those viable cells suspended in solution with NaCl and without NaCl, therefore, one condition with NaCl 0.85% was applied for other experiment. The probiotics are health promoting bacteria towards intestinal gut system and producers of many beneficial vitamins for the human health, such as vitamin K (menaquinones) and most of the water-soluble vitamins of group B, including biotin, nicotinic acid, folates, riboflavin, thiamine, pyridoxine, pantothenic acid and cobalamin (Rossi *et al.,* 2011). Probiotics help human health by providing beneficial outputs to gut, and, and breaking down some of undigested carbohydrate particles. Most of the enzymes are not encode by mammalian genome, and the structural polysaccharides present in plant materials needed to be digested.

The effect of $SO_2$ 1000 mg/L in *Lactobacillus acidophilus* KI, in intestinal a simulation model was studied and the results are shown in the figure 3.8. $SO_2$ didn't show any negative effect on bacterial cells viability during their incubation time for 24 hr. However, the positive controls had lost their viability after 6 hr interval of time. Sahadeva *et al.* (2011), investigated the effect of bile on *L. acidophilus* in commercial milk with different concentrations of 0%, 0.3% and 2.0% and keeping the pH up to 1.5, 3.0 and 7.2, for 24 hr in gastrointestinal tract simulation. pH up to 3.0 and 7.2 and bile at 2.0% did not inhibit the growth of the bacteria completely and there was still a high number of bacteria count (8.51 ± 0.03 log) observed in contrast with pH 1.5 in which bacteria didn't survived.

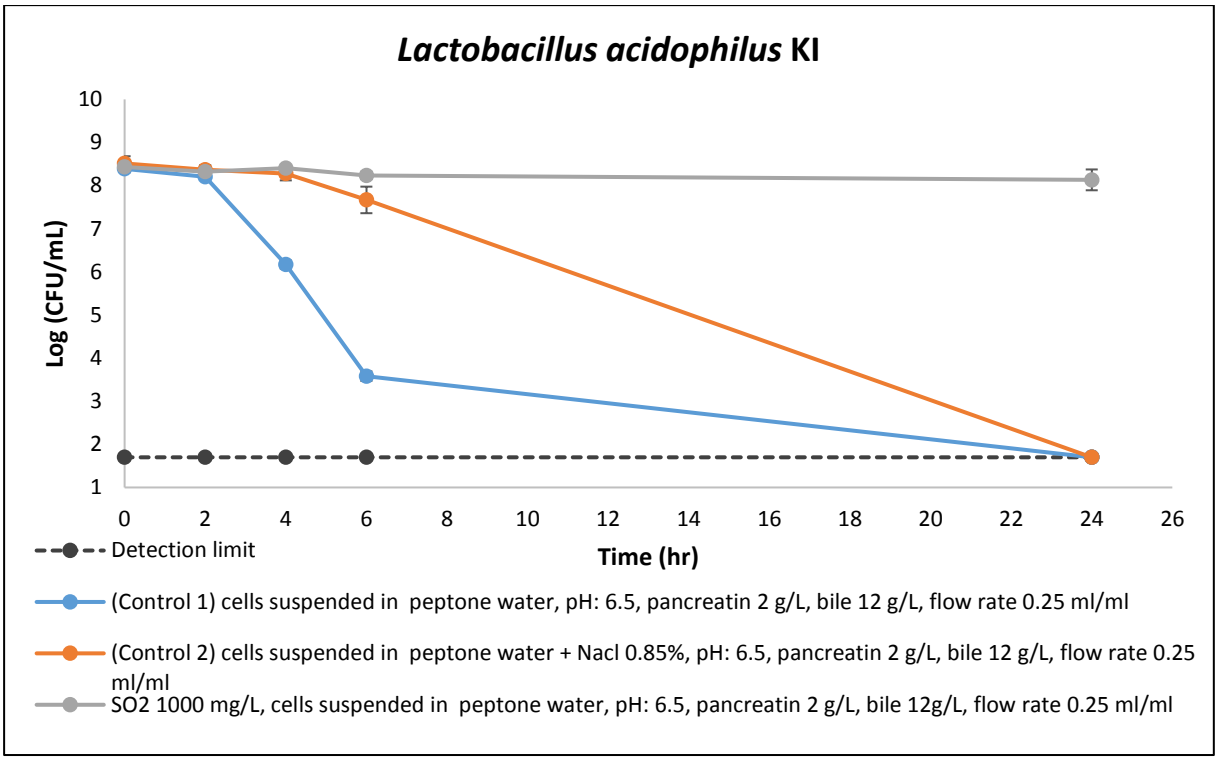



**Figure 3.8 -** Viable cell numbers of *Lactobacillus acidophilus* KI with $SO_2$ (1000 mg/L) and without $SO_2$ (control) in intestinal simulated simulation conditions.

In addition, inactivation of *Lactobacillus rhamnosus* R11 with $SO_2$ (1000 mg/L) in the intestinal simulation model was studied and results were obtained as can be seen in the figure 3.9. $SO_2$ had no inactivation impact on viability of bacterial strain during incubation time for 24 hr. But, positive controls, maintained their viability for up to 6 hr of incubation time and then lost their viability. According to Pitino *et al*. (2010) and Vamanu, (2017) which evaluated several strains of *L. rhamnosus* resistance to GIT conditions observed that most of them showed good survival abilities during duodenal digestion. This corelates directly with the synthesis of secondary metabolites (lactic acid and exopolysaccharides), which is a direct indicator of the capacity of strains tested in unfeasible environment at the gastric and small-intestine levels.

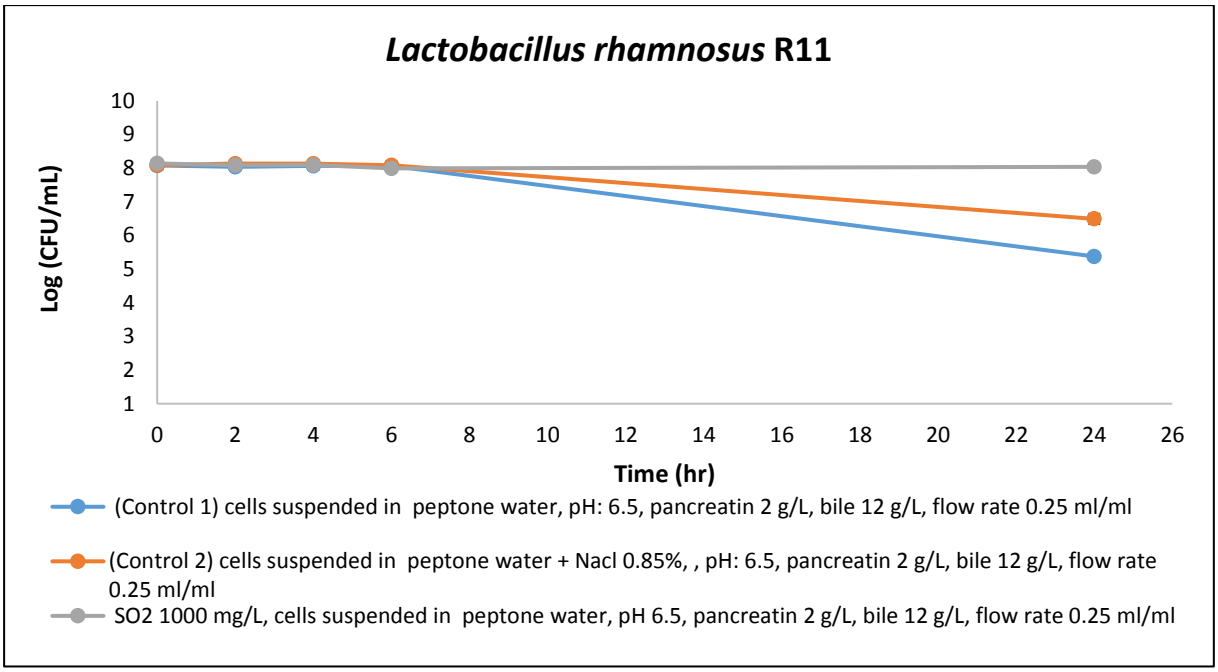



**Figure 3.9 -** Viable cell numbers of *Lactobacillus rhamnosus* R11 with $SO_2$ (1000 mg/L) and without $SO_2$ (control) in intestinal simulated simulation conditions.

Owing towards inactivation of *Lactobacillus plantarum* 299V with $SO_2$ (1000 mg/L) in intestinal simulation model and results were shown in the figure 3.10. Once again, the $SO_2$ had no adverse effect on the viability of such bacterial strain for 24 hr of their incubation time. And those viable cells number were remained the same as initially integrated, as seen from their viable cells count, in contrast However, the positive controls had shown a slight reduction in their viable cells number again after 6 hr of incubation as the previous bacterial strain. Zavisic *et al.* (2012), investigated the effect of bovine bile salts (0.5%) in artificial gastrointestinal simulation system, when those bacterial cells of *L. plantarum* G1 and *L. casei* G3. These strains were exposed to, while suspended in buffer phosphate salt solution at pH 8.0 and then incubated at 37$^{o}$C for 2 hr. There was a slight decrease in viable cells of *L. plantarum* G1 of about 0.45 log CFU/mL and *L. casei* G3 of about 0.22 log CFU/mL, which expedites the high degree survival of the isolates in the solution containing 0.5% bovine bile salts.

Barbosa *et al.* (2016), confirmed that there was a reduction of approximately 2 log from $10^9$ to $10^7$ CFU/mL for *Lactobacillus plantarum* 299V and *Pediococcus acidilactici* HA-6111-2 during both of the quick simulated digestion for 2 hr after bile salts addition and long-simulated digestion for 12 months of storage in orange juice. Thus, potentially exerting a beneficial effect on health of the consumers. *L. plantarum* 299V and *Lactobacillus* strains showed that, they can retain their viability for prolonged time and having survival capability of the passage, conveying colonization in the gastrointestinal tract (De Vries *et al.,* 2006).

Furthermore, *L. plantarum* 299V and LC 660 were exposed to different percentage level of increasing concentration of bovine bile salts (Ox gall) with 0.5%, 1.0%, 1.8%, and 3.6% (W/V) on their maximum growth rate and *L. plantarum* 299V had the best ability to grow in Ox gall-supplemented culture broth with relative growth rates of 85.5% to 97.1%. While *L. plantarum* LC 56 was the most sensitive strain towards bile salts, which had grown with relative growth rates of 19.9 to 58.2% (Hamon *et al.,* 2011).

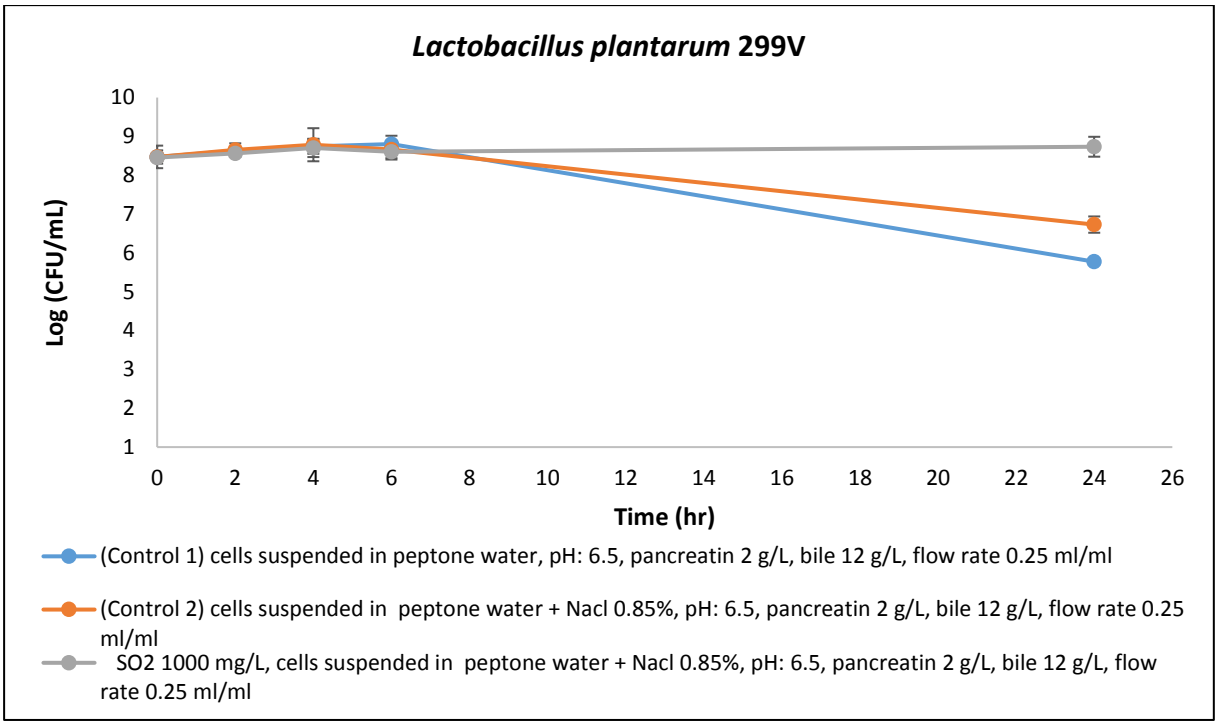



**Figure 3.10 -** Viable cell numbers of *Lactobacillus plantarum* 299V with $SO_2$ (1000 mg/L) and without $SO_2$ (control) in intestinal simulated simulation conditions.

Proceeding to bifidobacterial strains the inactivation of *Bifidobacterium animalis* Bo with $SO_2$ 1000 mg/L in intestinal simulation model was studied. Results were obtained and are depicted in the figure 3.11. $SO_2$ had somehow an inactivation effect upon viability of the bacterial cells during their incubation time of 24 hr. Though, the effect was not much intensive, since there was a minor decrease of about 1.9 log cycle in viable cells numbers. But positive controls as well did not maintain their cells viability and after 6 hr of incubation, there was a reduction in the viable cell's numbers. Madureira *et al*. (2005), studied *B. animalis* Bo, when incorporated in whey cheese and exposed to gastrointestinal simulated condition, and observed that bile salts had no such detrimental effect on viability of the *B. animalis* Bo, and viable cells remained constant until the end of the incubation period. And, this was the most resistant bacterial strain among several studied towards bile salts of with a death rate of only 0.0061 log (cfu $g^{-1}$) $min^{-1}$.

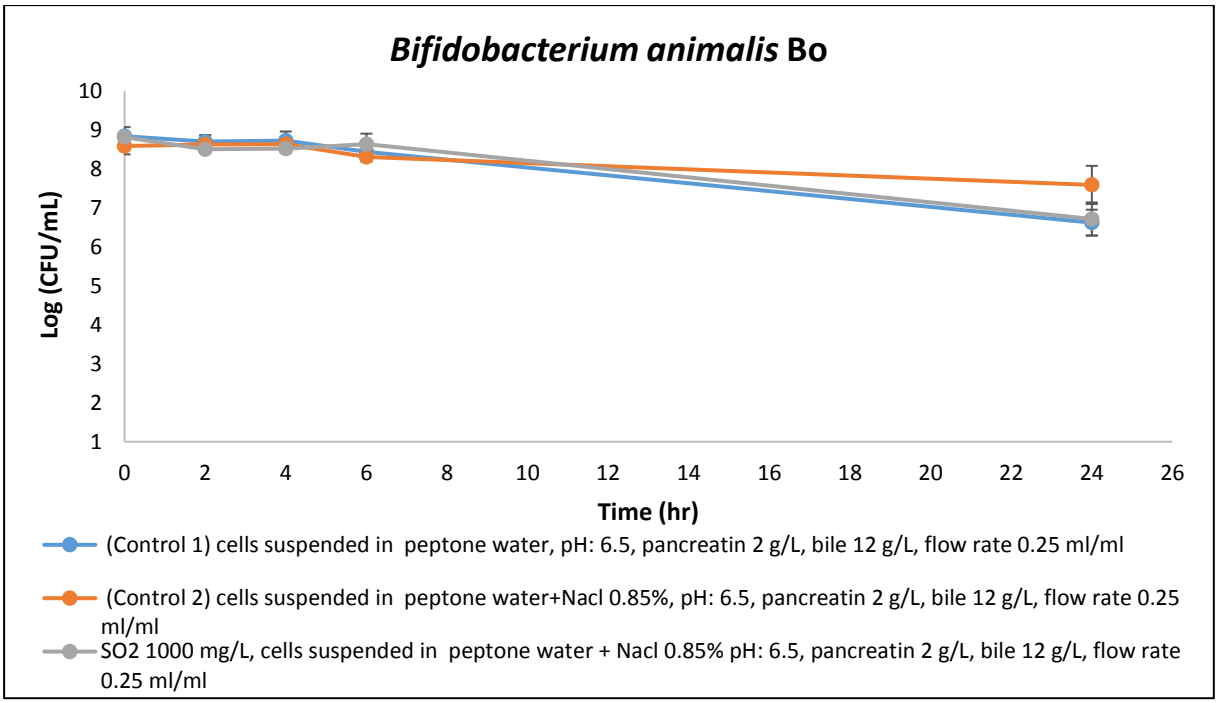



**Figure 3.11 -** Viable cell numbers of *Bifidobacterium animalis* Bo with $SO_2$ (1000 mg/L) and without $SO_2$ (control) in intestinal simulated simulation conditions.

In addition, the inactivation of *Bifidobacterium animalis* Bb12 with $SO_2$ 1000mg/L in the intestinal simulation model was studied and results were shown in figure 3.12. The $SO_2$ did not show any significant effect on the viability of bacterial cells during their incubation time of 24 hr. And the viable cells number remained the same. While, both positive controls showed reduction in the viability of the bacterial cells after 6 hr interval of time incubation. Madureira *et al*. (2005), concluded the effect of bile salts on *B. animalis* Bb12 into whey cheese, which was already pre-incubated in gastric juice for about 120 min. Firstly, there was a decrease of 1.5 log within the first 30 min and after the addition of bile salts, suffered an increase of approximately 1 log cycle in further 30 minutes. At last, the viable cell numbers had faced a reduction of 0.5 log over the remaining 60 min of period. Sahadeva *et al.,* (2011), revealed that bile salts had not any inhibitory effect on *Bifidobacterium*, when viable cells were exposed to bile salts of 0.3% and 2% at pH 1.5, 3.0, and 7.2, and incubated for 24 hr. The *Bifidobacterium* retained their viable cells numbers at 8 logs with pH 3.0 and 7.2 exposed to 0.3% of bile salt and at 7 logs with 2% of bile salts. But, at pH 1.5, bacteria cells didn't survive at all neither with 0.3% nor with 2% of the bile salts.

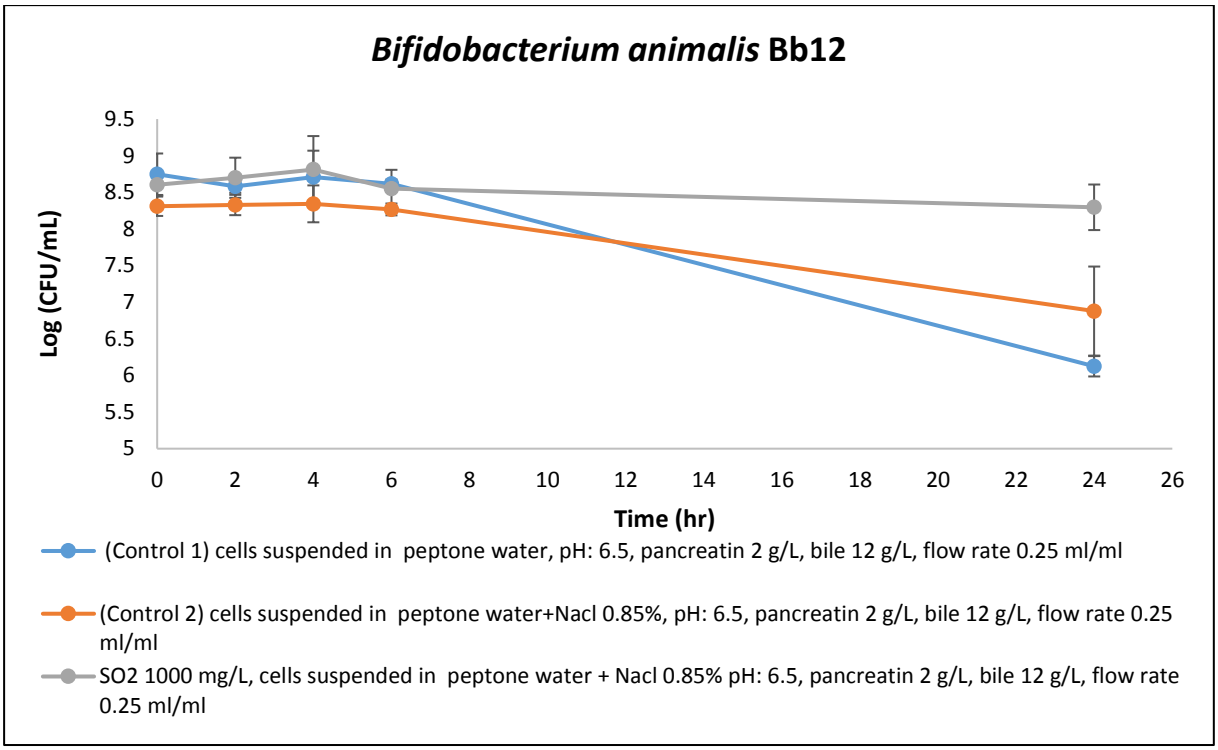



**Figure 3.12 -** Viable cell numbers of *Bifidobacterium animalis* Bb12 with $SO_2$ (1000 mg/L) and without $SO_2$ (control) in intestinal simulated simulation conditions.

### 3.3. Quantification of $SO_2$ in gastrointestinal tract (GIT) simulation model

Quantification of $SO_2$ throughout the simulated GIT was performed by HPLC and results are shown in the figure 3.13. For the three concentrations of (200 mg/L, 400 mg/L and 800 mg/L), no changes in each of GIT compartments occurred, except for the highest concentration at the end of the stomach that showed a slight increase. In fact, the pH had effect upon the various forms of $SO_2$, in which $SO_2$ remain mostly as bisulphite ($HSO_3^{-1}$), sulphites ($SO_3^{-2}$) and molecular sulphur dioxide ($SO_2.H_2O$) depending upon pH basic, strong basic and acidic (Fugelsang and Edwards, 2007), however there was not impact on final concentration throughout the GIT.

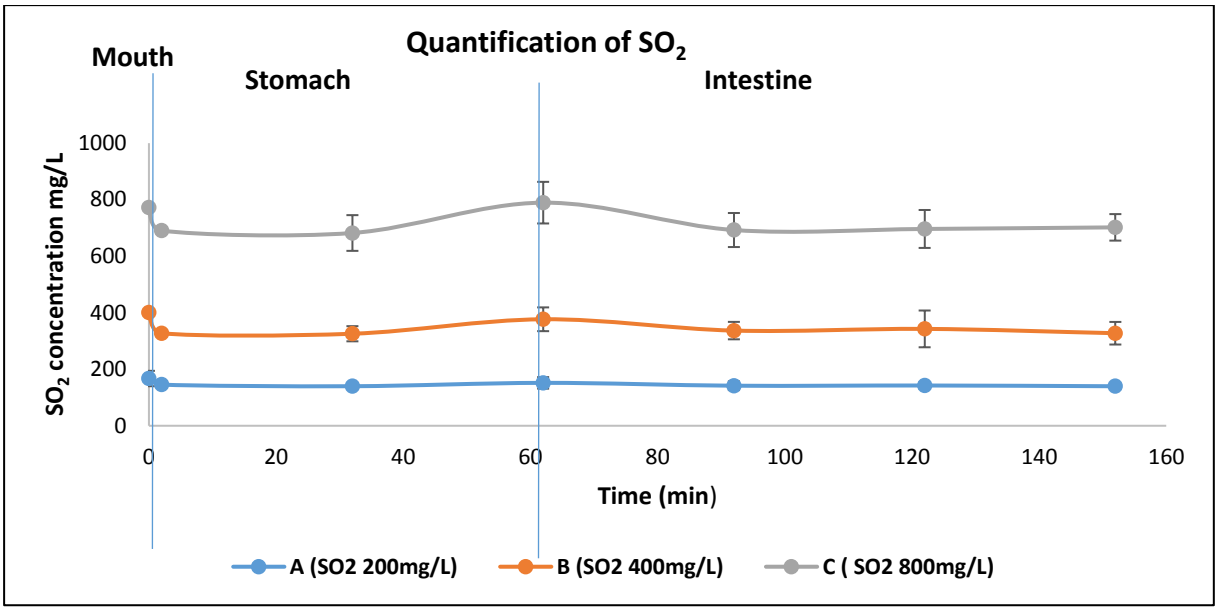



**Figure 3.13 -** Effect of gastric and intestinal enzymes at pH 6.9 (mouth), 2.5 (gastric) and 6.5 (intestine) on sulphur dioxide ($SO_2$) concentrations at different initial concentrations A: 200 mg/L, B: 400 mg/L and C: 800 mg/L throughout the simulated GIT.

### 3.4. Zeta potential and zeta size analysis of $SO_2$ through DLS

Analysis of effective diameter of particles (Zeta size) in test solution and of zeta potential (Zp) of $SO_2$ through the dynamic light scattering assays also revealed the existence of interactions. Effective diameter of complexes was variable depending on $SO_2$ structure and on its interaction with gastric and intestinal enzymes on their specific pH in different stages. The size of the particles ($SO_2$) in solution of ultra-pure water was increased, as they moved on and interacted with gastric juices; pepsin, forming $SO_2$-pepsin interaction and intestinal juices; bile and pancreatin, forming $SO_2$-bile-pancreatin interaction and formed a complex by agglomeration. The zeta potential was also monitored through dynamic light scattering assays (DLS) and it was revealed that, pH had significant effect upon $SO_2$ particles. The $SO_2$ has negative charge on its surface layer at pH 6.9 in mouth, while the effect changed towards positivity, when the environment changed to acidic pH (2.5) in the stomach. But, as the particles moved on towards intestine and the condition changed to basic environment at pH value 6.5, the charge on particles got more negative in overall gastrointestinal simulation. As can be seen from the figures 3.14 and 3.15, which had shown that pH has very prominent effect upon $SO_2$, which demonstrates that it may exist in three different forms, molecular $SO_2$ is also a neutral form when Zp value is 0 without any charge with the environment is acidic having pH 2.5 that correlates stomach condition, which is the most active form for antimicrobial activity. Then, the second form is $HSO_3^{-1}$ (bisulphites) and third form is $SO_3^{-2}$ (sulphites), which both exist at pH 6.0-6.5 having basic environment with negative charge, which correlates intestine (Fugelsang and Edwards, 2007).

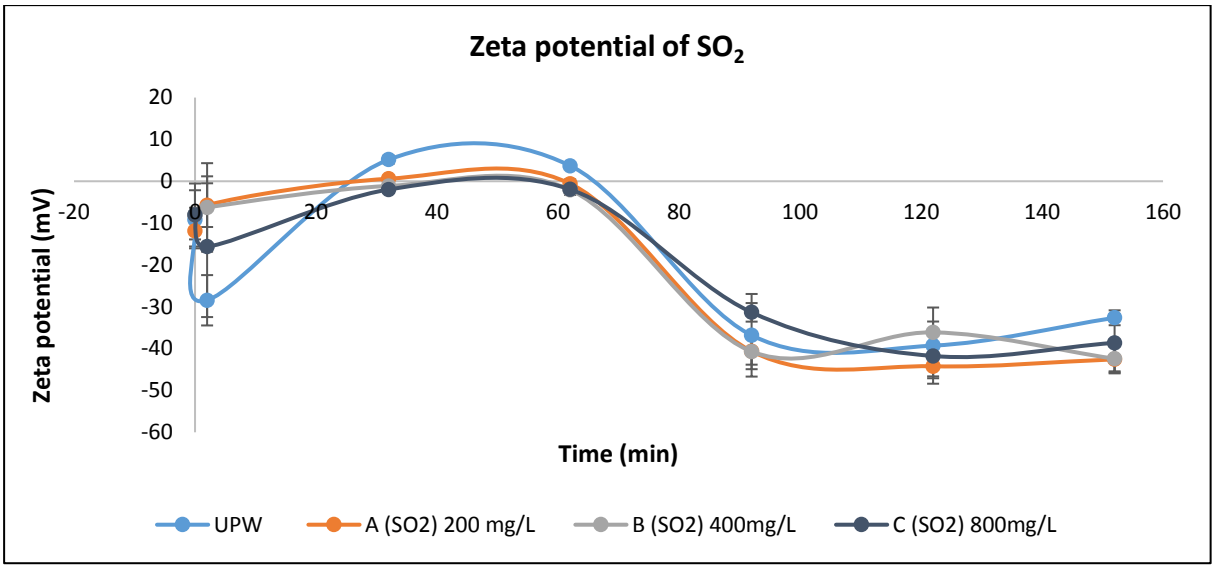



**Figure 3.14 -** Zeta potential of $SO_2$ quantification in gastrointestinal tract simulation (GIT) model throughout the time for three different concentrations of $SO_2$ dissolved in Ultra-pure water (UPW)

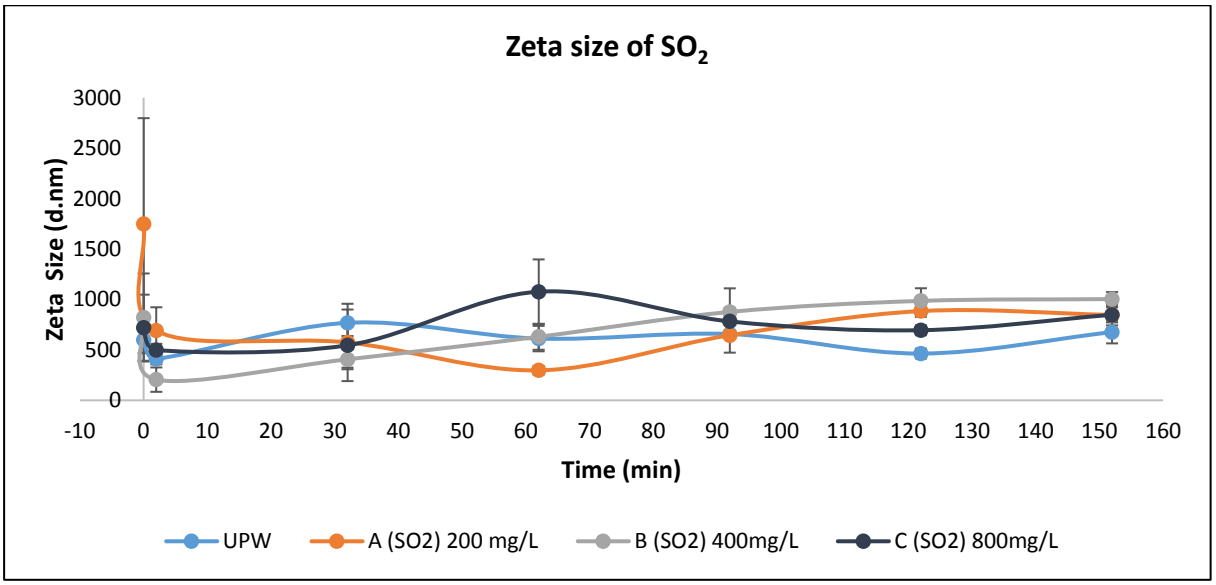



**Figure 3.15 -** Zeta size of $SO_2$ quantification in gastrointestinal tract simulation (GIT) mode throughout the time for three different concentrations of $SO_2$ dissolved in Ultra-pure water (UPW)

### 3.5. Effect of $SO_2$ on the metabolism of *Bifidobacterium animalis* Bo

The growth of *B. animalis* Bo is directly correlated with its metabolism during incubation time, in which acids production were affected related to its growth curves. The growth was inhibited somehow when growing with both parameters with $SO_2$ 1000 mg/L compared to a control without $SO_2$. *B. animalis* Bo without $SO_2$ showed a curve with normal growth rate reaching ca. 5 x $10^9$ CFU/mL of maximum growth in the stationary phase. When cultured with $SO_2$, the growth rate was affected and stationary phase started at 12 hr instead of 8 hr and the maximum growth in the stationary phase was reduced ca. 1 log cycle (ca. 5 x $10^8$ CFU/mL) and as can be seen the metabolism of *B. animalis* Bo was highly affected

by $SO_2$ (1000 mg/L) as confirmed by HPLC. $SO_2$ had significant negative effect upon glucose consumption in metabolism comparing with the positive control, as shown in the figure 3.16.

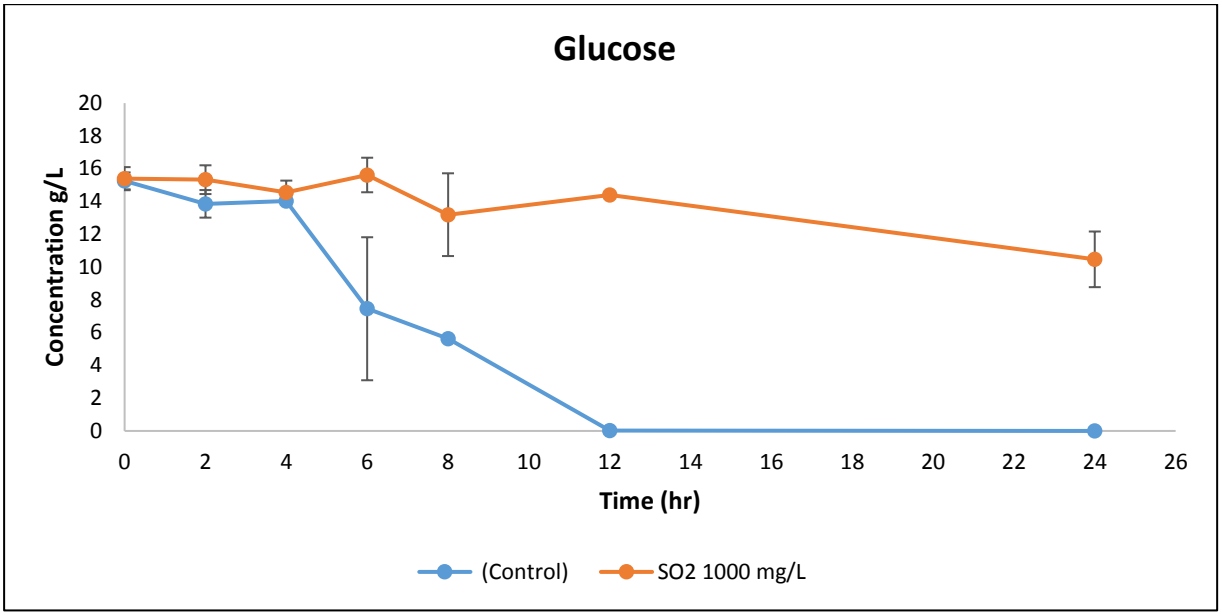



**Figure.3.16-** The effect of $SO_2$ (1000 mg/L) on the metabolism of *B. animalis* Bo concerning Glucose consumption.

The impact of $SO_2$ (1000 mg/L) upon organic acids production during metabolism of *B. animalis* Bo was also studied. The impact of $SO_2$ (1000 mg/L) on citric acid production was investigated and compared with positive control. There was no significant effect in this acid production until 12 hr of incubation. But, after 12 hr, the acid production was greatly affected and there was about 0.845 g/L decrease observed which becomes 50% of inhibition in citric acid production during its metabolism as shown in the figure 3.17.

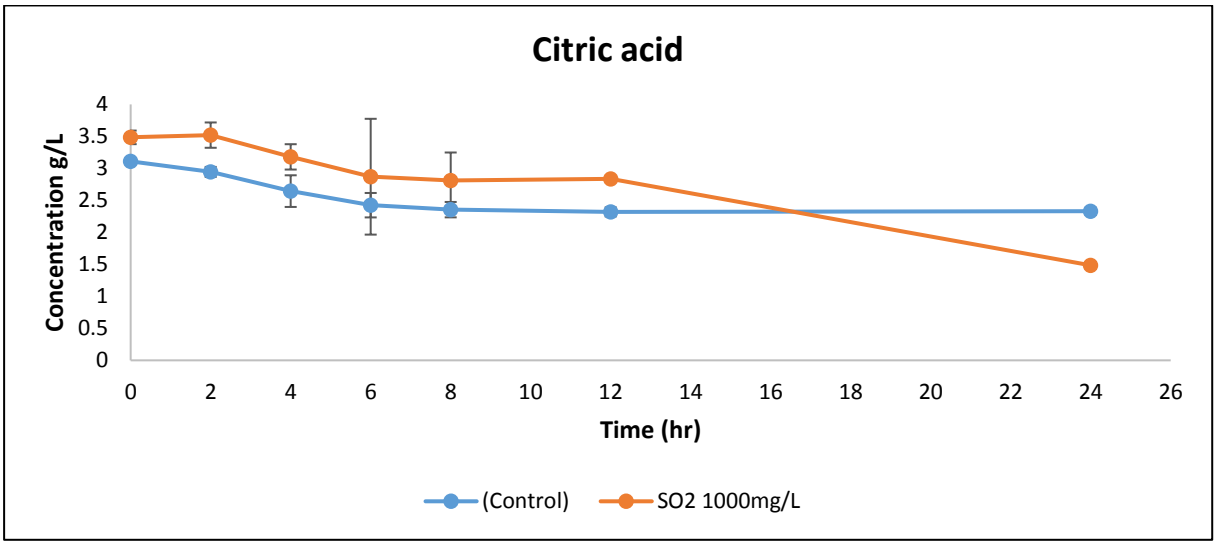



**Figure 3.17 -** The effect of $SO_2$ (1000mg/L) on the metabolism of *B. animalis* Bo concerning citric acid production.

The impact of $SO_2$ (1000 mg/L) upon lactic acid production was greatly affected during its metabolism, compared to the positive control. The acid production was hardly reached up to 2.685 g/L in 12 hr of incubation as compared to control, which had reached up to 20.36 g/L within same time. Afterwards, from 12 hr to 24 hr of incubation there was 5.6 g/L increase observed from 2.685 g/L to 8.27 g/L in acid production. So, the control reached 21.05 g/L within 24 hr of its incubation time as shown in the figure 3.18.

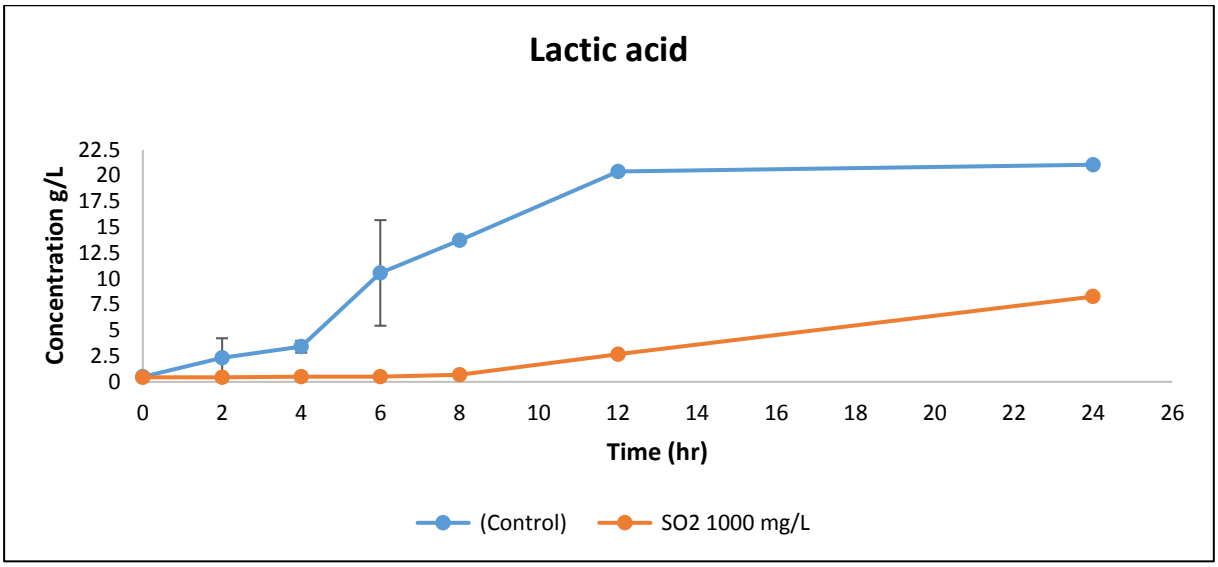



**Figure 3.18 -** The effect of $SO_2$ (1000 mg/L) on the metabolism of *B. animalis* Bo concerning lactic acid production.

The production of succinic acid with $SO_2$ (1000 mg/L) was slightly affected during its metabolism, when compared the positive control (see figure 3.19).

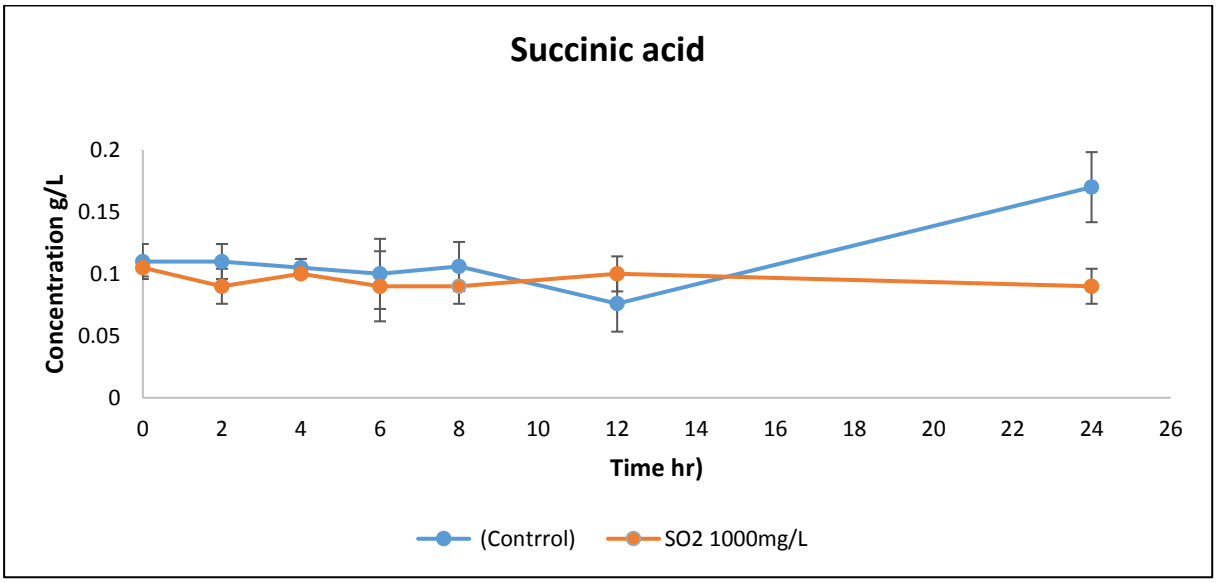



**Figure 3.19 -** The effect of $SO_2$ (1000 mg/L) on the metabolism of *B. animalis* Bo concerning succinic acid production.

The $SO_2$ (1000 mg/L) applied during did not affect acetic acid production compared to the positive control, since there was no difference in acetic acid production throughout incubation time for 24 hr as can be seen in figure 3.20.

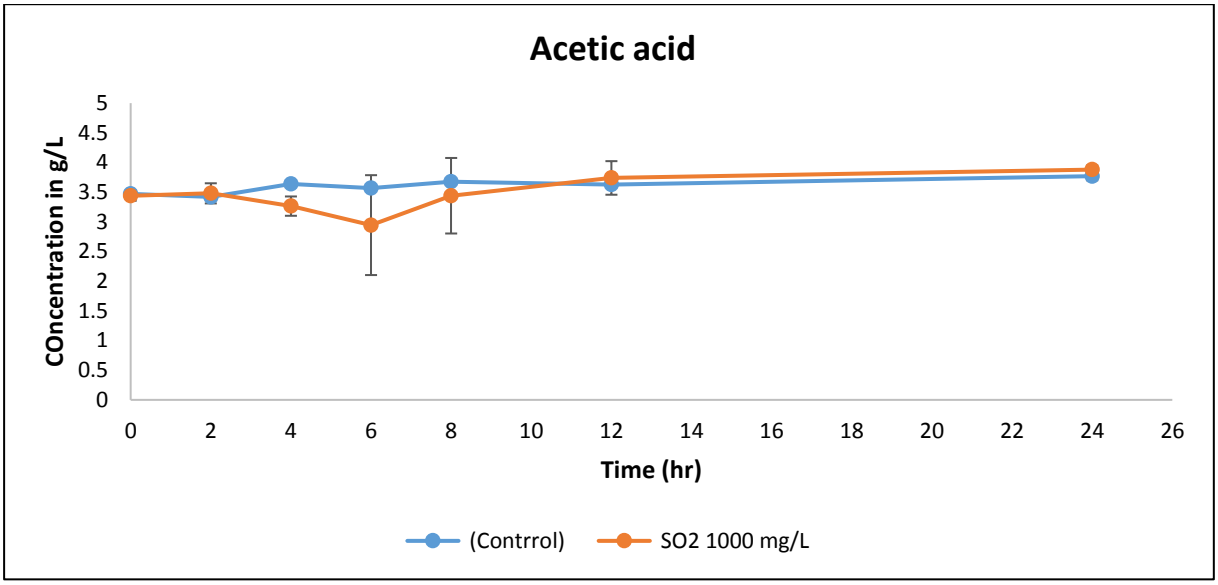



**Figure 3.20 -** The effect of $SO_2$ (1000 mg/L) on the metabolism of *B. animalis* Bo concerning acetic acid production.

The propionic acid production was not affected during microbial metabolism by $SO_2$ (1000 mg/L), when compared with positive control. During acid production, there was no variance observed between the test compound and the control for 24 hr of incubation time(see figure 3.21).

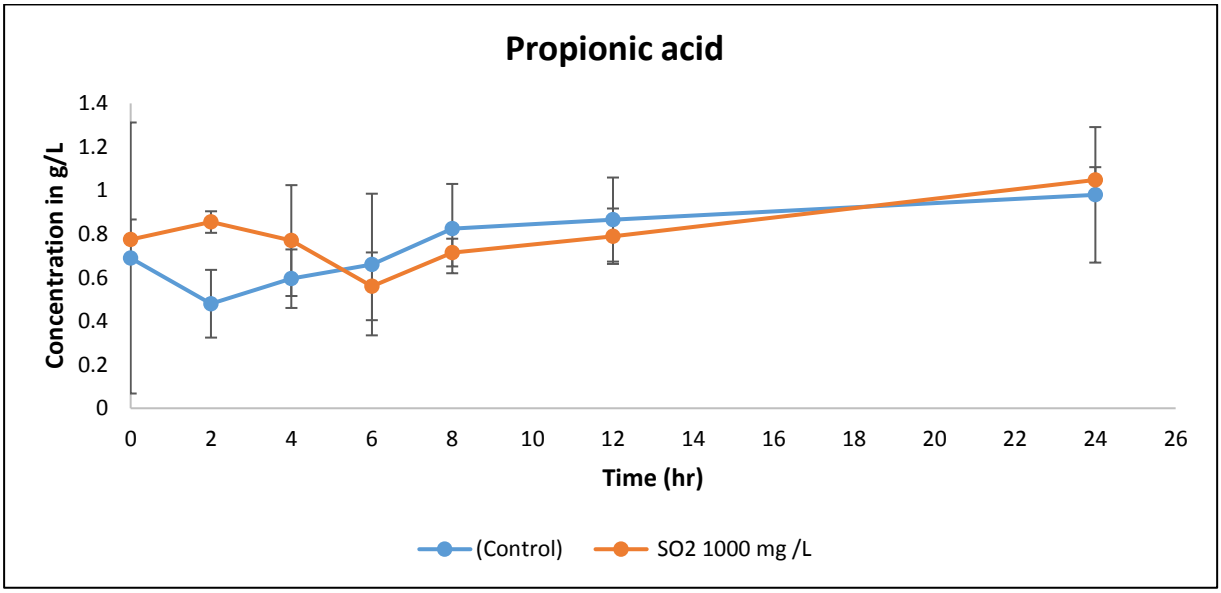



**Figure 3.21 -** The effect of $SO_2$ (1000 mg/L) on the metabolism of *B. animalis* Bo concerning propionic acid production.

The butyric acid production was not affected by $SO_2$ (1000 mg/L) during microbial metabolism throughout incubation time for 24 hr as can be seen in the figure 3.22. However, the control showed a slight reduction mainly after 8 h of incubation. while having positive control, and even though, there was no evidence of the produced acids.

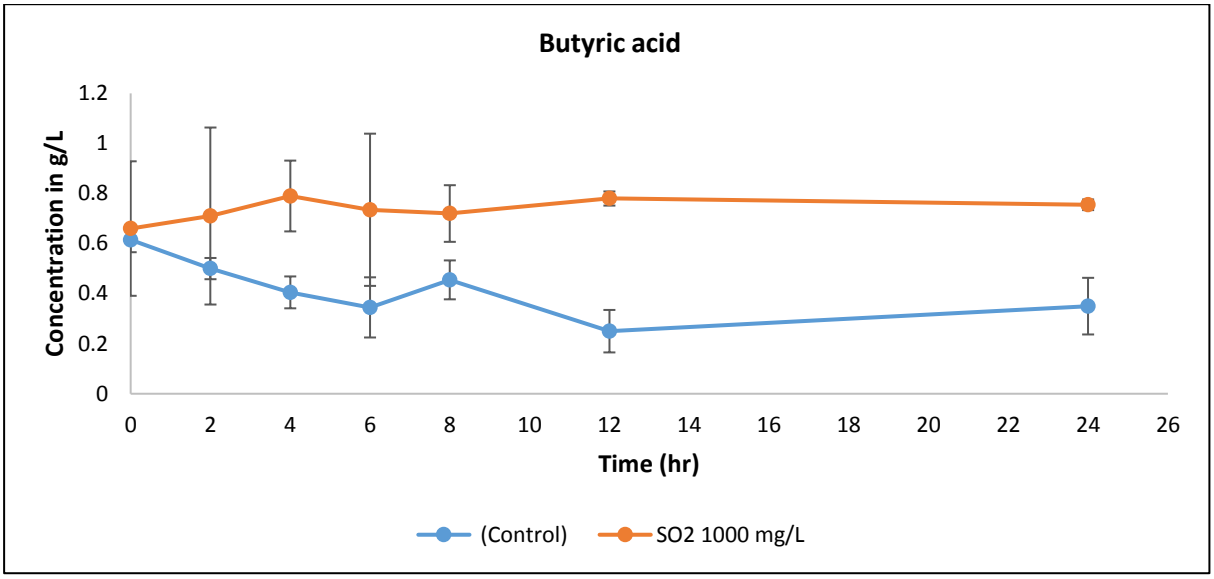



**Figure 3.22 -** The effect of $SO_2$ (1000 mg/L) on the metabolism of *B. animalis* Bo concerning butyric acid production.

## 4. Main Conclusions

The present thesis research work aimed to see the effect of sulphur dioxide ($SO_2$) as an antimicrobial agent on pathogenic and probiotic bacterial strains regarding their inactivation in the gastrointestinal simulation model.

Growth curves of probiotic and pathogenic bacterial strains with $SO_2$ at concentrations of 500 mg/L and 1000 mg/L were performed, and it was observed that $SO_2$ at concentration 500 mg/L had no significant negative effect on probiotic or pathogenic bacterial strains. However, $SO_2$ at concentration of 1000 mg/L had adverse effect on growth curve mainly upon *B. animalis* Bo. For pathogenic bacterial strains, $SO_2$ at concentration of 1000 mg/L showed negative effect on the growth of *L. monocytogenes*, *E. coli* and *S. enteritidis*.

Afterwards, the growth curve of *B. animalis* Bo with $SO_2$ at 1000 mg/L was established by enumeration of viable cells numbers. It was confirmed that $SO_2$ had slight negative effect upon *B. animalis* Bo since the bacterial cells number reduced ca. 1 log cycle.

During inactivation of pathogenic bacterial strains with $SO_2$ at 1000 mg/L under gastric conditions, it was concluded that the presence of $SO_2$ had no any negative effect on them. On the other hand some positive control didn't survive, since they lose their viability for longer periods. Furthermore, during inactivation of probiotic bacterial strains with $SO_2$ at 1000 mg/L in intestinal simulation, it was concluded that there was a very small reduction in bacterial cells number after their viable cells count. Though, some controls had also lost certain viability after certain interval of incubation time. So, the presence of high concentration of $SO_2$ may protect the bacterial inhibition under GIT conditions.

During quantification of $SO_2$ through *in vitro* gastrointestinal tract model, it was also confirmed that no negative effect of gastric and intestinal juices was observed on $SO_2$ concentrations for all the tested concentrations - 200 mg/L, 400 mg/L and 800 mg/L.

The size of $SO_2$ particles and their charge changed during complete analysis of GIT passage since they interacted with gastric and intestinal juices. Since the pH did affect the charged particles was also observed, the dynamic light scattering assay was also studied through. The size of the test particles ($SO_2$) increased passing throughout the passage of gastric and intestinal stages due to the interaction with pepsin, bile and pancreatin to form a complex structure. The zeta potential was also studied, and the effect of pH was determined to understand how it triggered the particles from negativity towards positively condition and from positivity towards negatively condition in gastrointestinal tract simulation model.

The metabolism of *B. animalis* Bo with $SO_2$ (1000 mg/L) has shown conclusive results demonstrating that $SO_2$ had strictly inhibited the glucose consumption.

During metabolism of *B. animalis* Bo, organic acids production was also monitored and the effect of $SO_2$ (1000 mg/L) was studied. It was concluded that, $SO_2$ had been inhibiting mainly the production of lactic acid and citric acid, while acetic and propionic acids production were not inhibited. Though, succinic acid production was somehow inhibited, and there was no butyric acid production observed.

The global results conclude that $SO_2$ had not much potential for its antimicrobial activity upon probiotic bacteria avoiding its inhibition throughout GIT and confirming no detrimental effect.

Concerning pathogenic bacteria the $SO_2$ promoted a positive effect inducing some inactivation that is strain dependent.

**5. Future work**

Based on the results it is important to understand in future studies the effect of $SO_2$ on the protection of bacteria throughout GIT, since the bacteria in the controls have been decreased mainly for some of the pathogens.

Sometimes, when $SO_2$ is proceeding towards gut, and before it approach the gut, it may be degraded by other pathogenic bacteria in stomach or may also be transformed into another form and made them unavailable for the desired bacterial species or strains, which needs to investigate them more deeply, whether $SO_2$ approach to gut in real state or not.

It should not to be underestimate that, $SO_2$ may also leak out and absorbed towards gut walls and make it unavailable to desired bacterial strains with exact concentration in real gastrointestinal system. We must evaluate such point for having practical experience *in vitro*.

According to the present experience performed, those bacterial strains were directly suspended in sulphur dioxide solution ($SO_2$ 1000 mg/L) for their inactivation phenomenon. But, in the future, the bacteria cells ought to be exposed towards different kind of food matrices such as meat, canned olives, fruits, dried fruits etc. which are already preserved with $SO_2$ for their inactivation procedure in the gastrointestinal tract simulation model to see the differences in both the conditions,

## 6.0 Appendixes

### 6.1. Appendix 1-Calibration curves

#### 6.1.1. Standard calibration curve made for $SO_2$ quantification in GIT

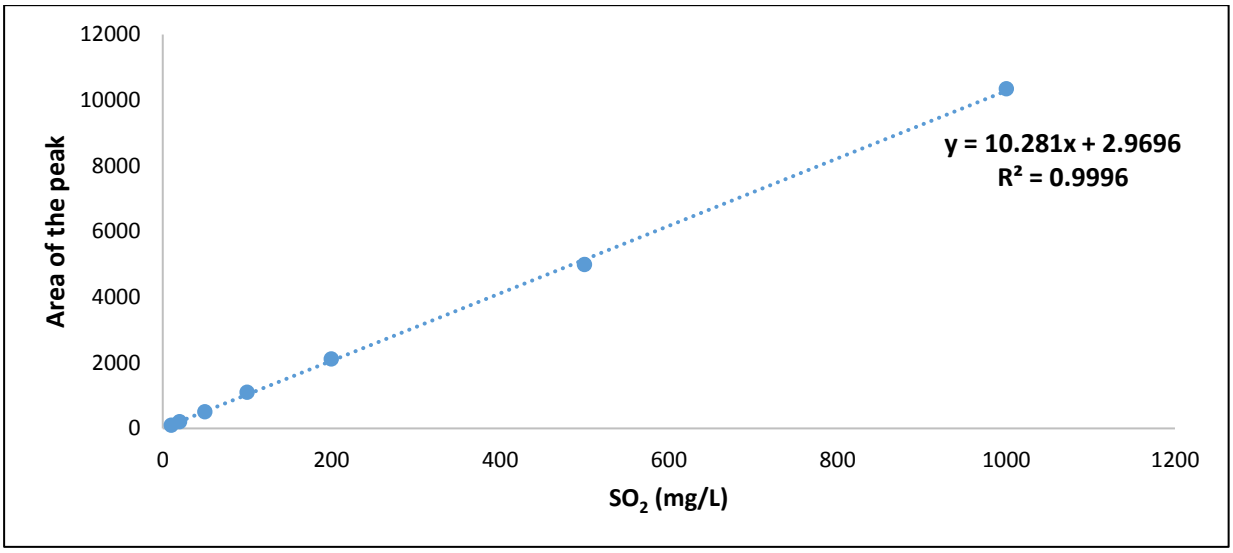



**Figure 6.1-** Calibration curve obtained by HPLC, using sulphur dioxide standard solutions, in order to quantify sulphur dioxide in test solutions in (GIT).

#### 6.2.1 Glucose calibration curve

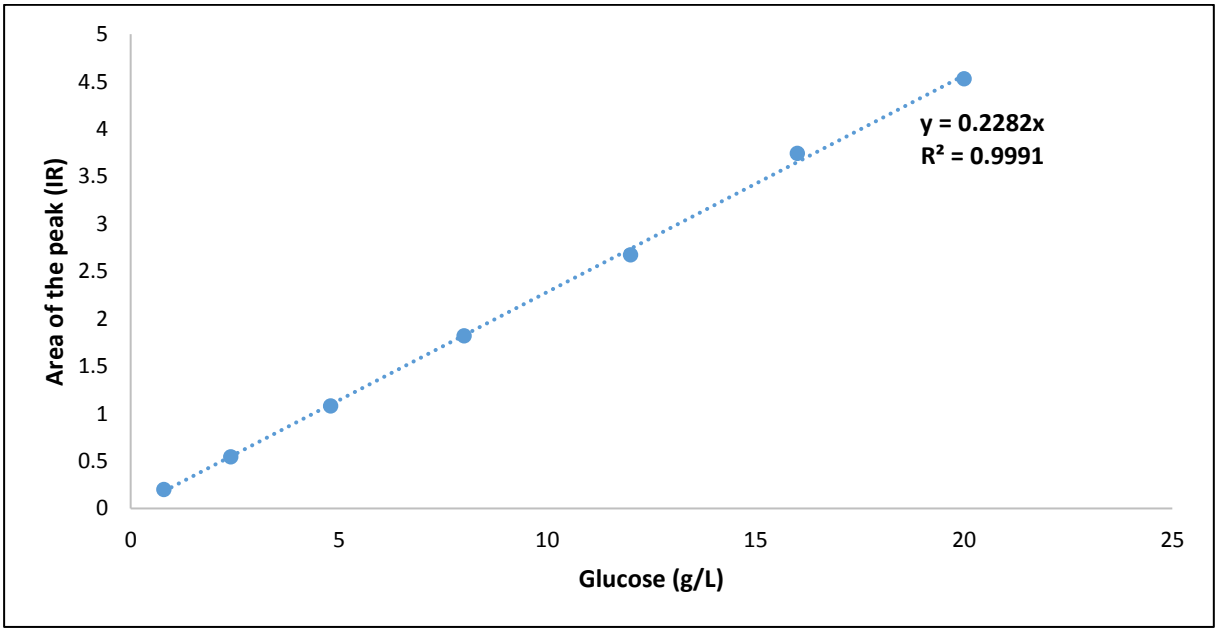



**Figure 6.2-** Calibration curve obtained by HPLC, using glucose standard solutions, in order to quantify glucose in test solutions.

### 6.3.1 Citric acid calibration curve

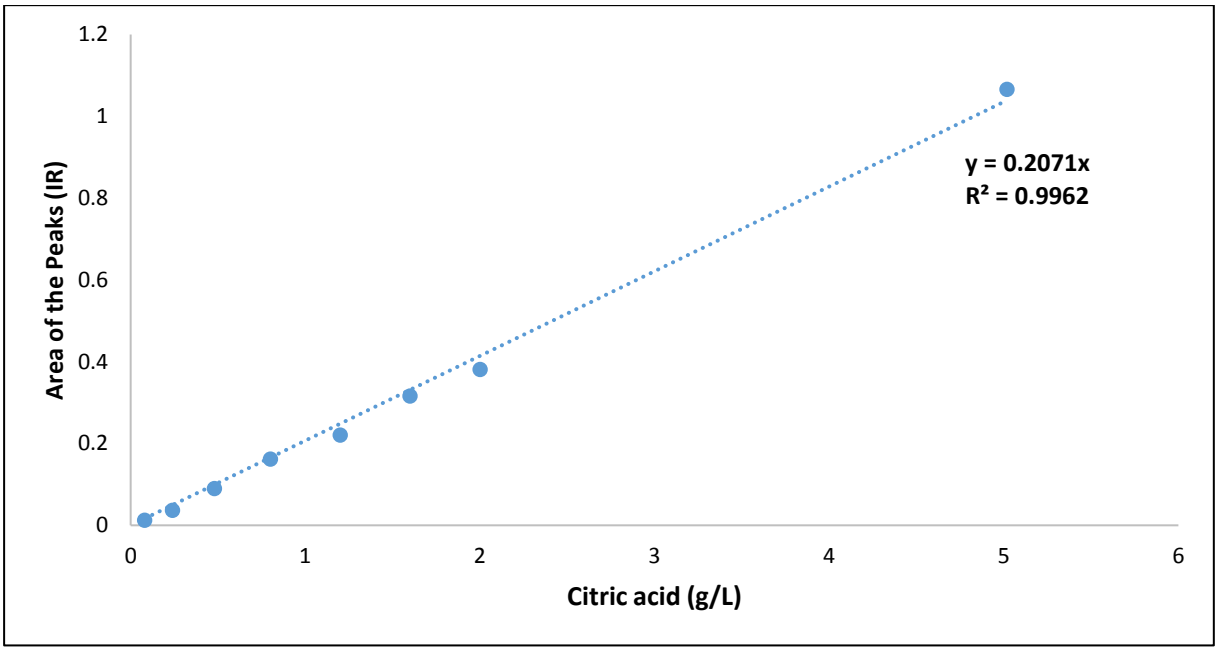



**Figure 6.3-** Calibration curve obtained by HPLC, using citric acid standard solutions, in order to quantify citric acid in test solutions.

### 6.3.2 Succinic acid calibration curve

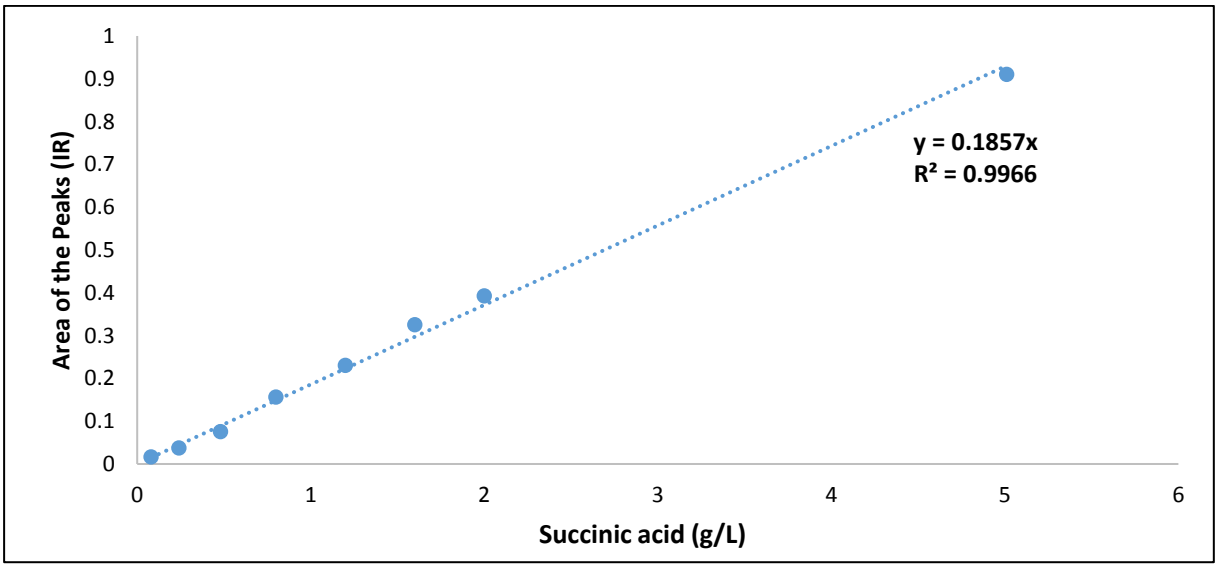



**Figure 6.4-** Calibration curve obtained by HPLC, using succinic acid standard solutions, in order to quantify succinic acid in test solutions.

### 6.3.3 Lactic acid calibration curve

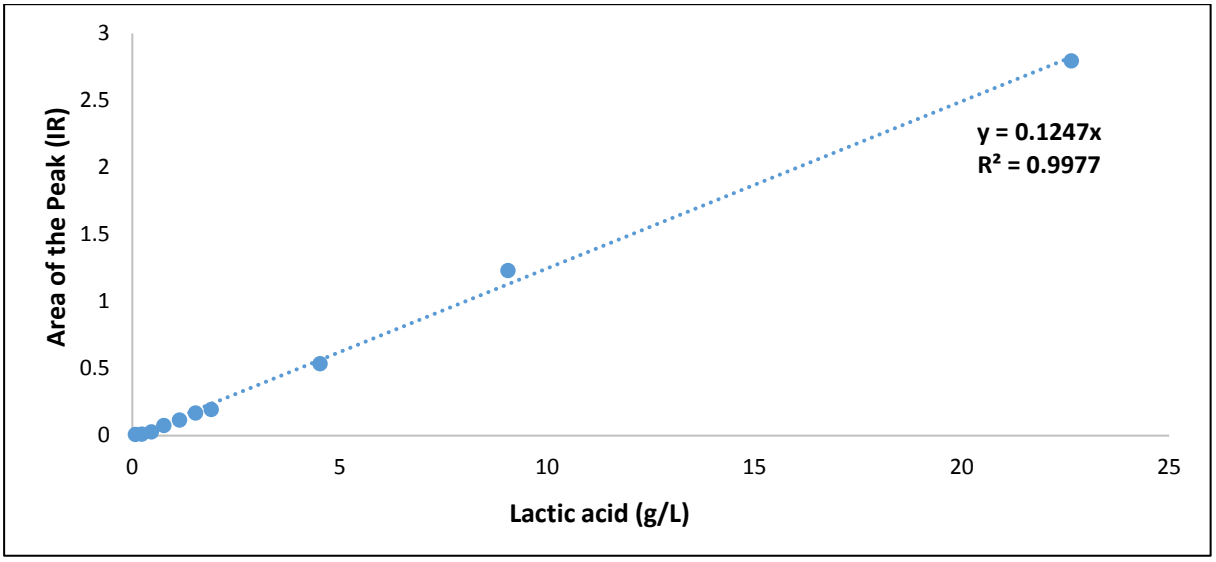



**Figure 6.5-** Calibration curve obtained by HPLC, using lactic acid standard solutions, in order to quantify lactic acid in test solutions

### 6.3.4 Acetic acid calibration curve

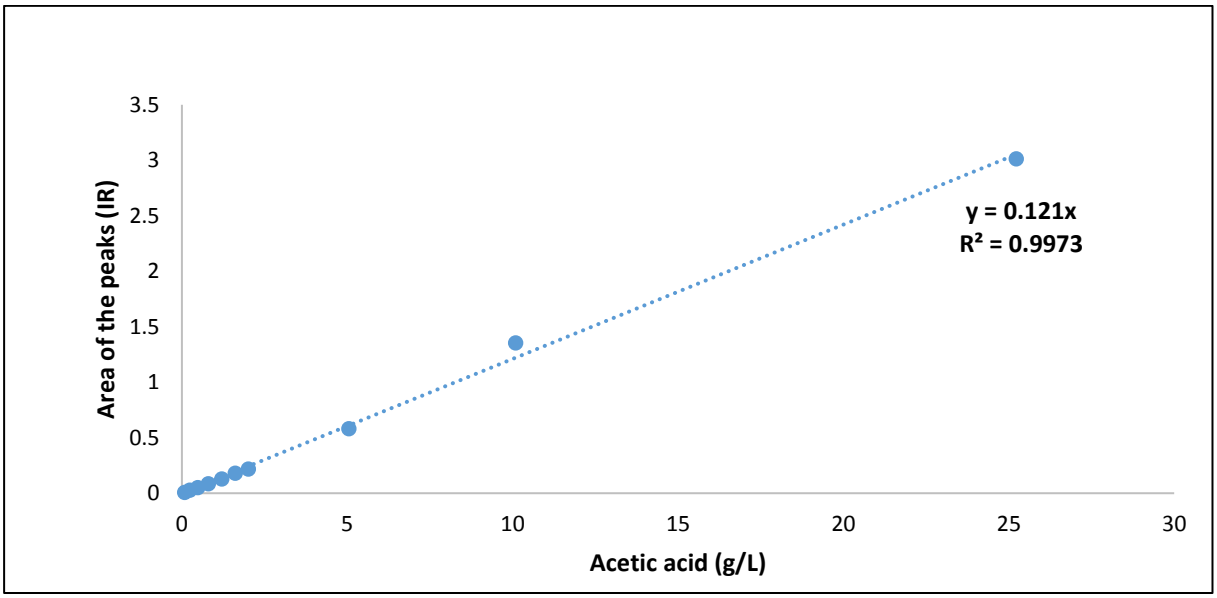



**Figure 6.6-** Calibration curve obtained by HPLC, using acetic acid standard solutions, in order to quantify acetic acid in test solutions.

### 6.3.5 Propionic acid calibration curve

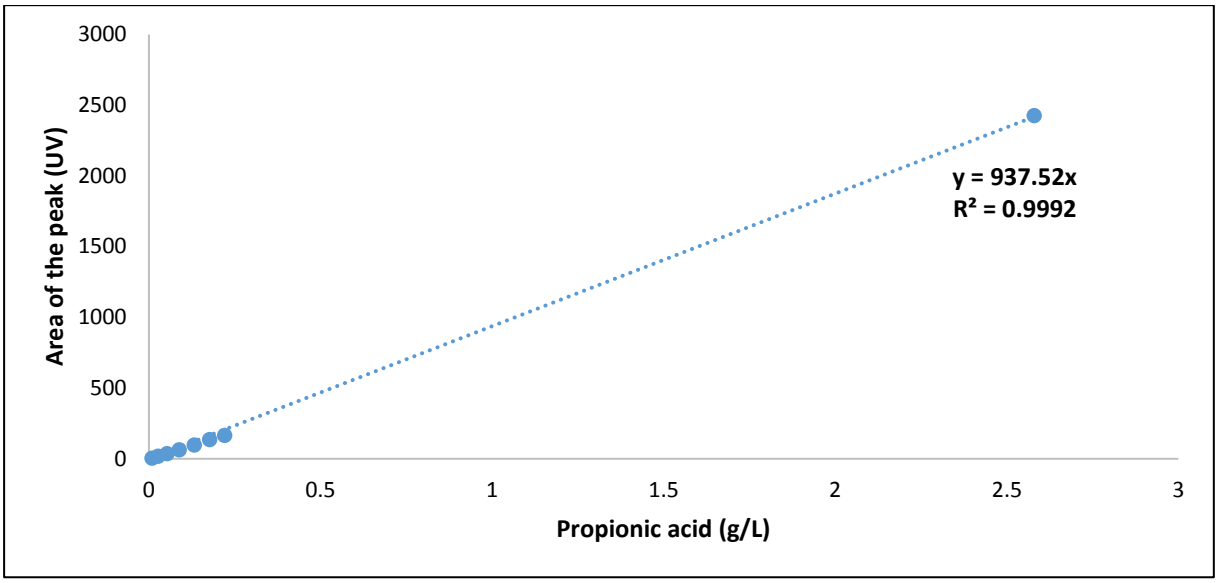



**Figure 6.7-** Calibration curve obtained by HPLC, using propionic acid standard solutions, in order to quantify propionic acid in test solutions.

### 6.3.6 Butyric acid calibration curve

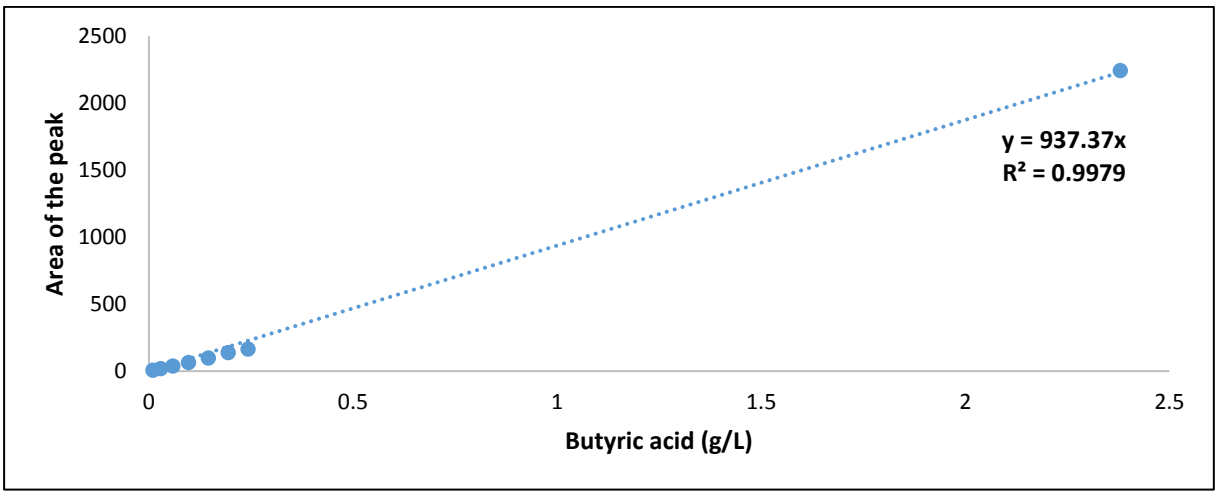



**Figure 6.8-** Calibration curve obtained by HPLC, using butyric acid standard solutions, in order to quantify butyric acid in test solutions.

## 7. Bibliographic References


1. Barbosa, J., Borges, S., Teixeira, P. 2016. Effect of different conditions of growth and storage on the cell counts of two lactic acid bacteria after spray drying in orange juice. *Beverages* 2(2):8.
2. COMMENDIUM OF INTERNATIONAL METHODS OF ANALYSIS-OIV. Maximum acceptable limits of various substances contained in wine www.oiv.int/public/medias/2601/oiv-ma-c1-01.pdf
3. Ceuppens, S., Uyttendaele, M., Hamelink, S., Boon, N., and Van de Wiele, T. 2012. Inactivation of *Bacillus cereus* vegetative cells by gastric acid and bile during in vitro gastrointestinal transit. *Gut Pathogens* 4(1):11.
4. Ceuppens, S., Uyttendaele, M., Drieskens, K., Rajkovic, A., Boon, N., and Van de Wiele, T.2012. Survival of *bacillus cereus* vegetative cells and spores during in vitro simulation of gastric passage. *Journal of Food Protection* 75(4):690-694.
5. Chen, L., Borba, B. D., Rohrer, J. 2015. Determination of total and free sulfite in food and beverages. Thermo Fisher Scientific, Sunnyvale, CA, USA, App. Note. 54.
6. Claudia, R. C., Francisco, J. C. 2009. Application of flow injection analysis for determining sulphites in food and beverages: A review. *Food Chemistry* 112(2):487-493.
7. De Vries, M. C., Vaughan, E. E., Kleerebezem, M., de Vos, W. M. 2006. *Lactobacillus plantarum* survival, functional and potential probiotic properties in the human intestinal tract. *International Dairy Journal* 16(9):1018-1028.
8. DiPersio, P. A., Kendall, P. A., Sofos, J. N. 2004. Inactivation of *Listeria monocytogenes* during drying and storage of peach slices treated with acidic or sodium metabisulfite solutions. *Food Microbiology* 21(6):641-648.
9. European commission regulation of the European Parliament and of the Council by establishing a Union list of food additives https://www.fsai.ie/uploadedFiles/Reg1129_2011.pdf.
10. European Commission. Commission regulation (EU) No 1129/2011. amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. Off J Eur Union. 2011;(L295/1)
11. Engelkirk, P. G., Duben-Engelkirk, J. L., Wilson Burton, G. R. 2011. Burton's Microbiology for the Health Sciences, revised edition. Lippincott Williams & Wilkins, pp.123.
12. Freedman, B. J., 1980. Sulphur dioxide in food and beverages: Its use as a preservative and its effect on Asthma. *British Journal of Diseases of the Chest* 74:128.
13. Fernandes, J. C., Tavaria, F. K., Soares, J. C., Ramos, O. S., Monteiro, M. J., Pintado, M. E., Malacata, F. X. 2008. Antimicrobial effects of chitosans and chitooligosaccharides, upon *Staphylococcus aureus* and *Escherichia coli*, in food model systems. *Food Microbiology* 25(7):922-928.
14. Fugelsang, K. C., Edwards, C. G. 2007, Wine microbiology, practical applications and procedures. Preservatives and sterilants 2nd edition, pp. 66-67.
15. Garcia-Fuentes, A. R., Wirtz, S., Vos, E., Verhagen, H. 2015. Short review of sulphites as food additives. *European Journal of Nutrition & Food Safety* 5(2):113-120.

16. Hui, Y. H., Evranuz, E. O. 2016. HANDBOOK OF VEGETABLES PRESEVATION AND VEGETABLES PROCESSING, 2nd edition, CRC Press, Taylor and Francis Group, pp. 312.
17. Hamon, E., Horvatovich, P., Izquierdo, E., Bringel, F., Marchioni, E., Aoude-Werner, D., Ennahar, S. 2011. Comparative proteomic analysis of Lactobacillus *plantarum* for the identification of key proteins in bile tolerance. *BMC Microbiology* 11:63.
18. Jay, J. M. 1998. Modern Food Microbiology, fifth edition. Aspen Publishers, Inc. Gaithersburg, Maryland, pp. 278.
19. Kocamaz, E., Adiguzel, E., ER, B., Gundogdu, G., Kucukatay, V. 2012. Sulfur leads to neuron loss in the hippocampus of both normal and SOX-deficient rats. *Neurochemistry International* 61(3):341-346.
20. Koseki, S., Mizuno, Y., Sotome, I. 2010. Modeling of Pathogen survival during Simulated Gastric Digestion. *Applied and Environmental Microbiology* 77(3):1021-1032
21. Taylor, L. S., Highley, A. N., Bush, K. R. 1986. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity, and hypersensitivity, Advance in food research, Vol.30. Academic press, Inc.
22. Lester, M. R. 1995. Sulfite sensitivity: significance in human health. *Journal of the American College of Nutrition* 14(3):229-232.
23. Luque de Castro, M. D., Gonzalez-Rodriguez, J., Perez-Juan, P. 2005. Analytical methods in wineries: Is it time to change? *Food reviews international* 21(2):231-265.
24. Londono, S. C., Hartnett, H. E., Williams, L. B. 2017. Antibacterial Activity of Aluminium in Clay from the Colombian Amazon. *Environmental Science & Technology* 51(4):2401-2408.
25. Leyer, G. J., and Johnson, E. A. 1993. Acid adaptation induces cross-protection against environmental stresses in *Salmonella typhimurium*. *Applied and Environmental Microbiology* 59(6):1842-1847.
26. Madureira, A. R., Amorim, M., Gomes, A. M., Pintado, M. E., Malcata, F. X. 2011. Protective effect of whey cheese matrix on probiotic strains exposed to simulated gastrointestinal conditions. *Food Research International* 44(1):465-470.
27. Madureira, A. R., Periera, C. I., Truszkowska, K., Gomes, A. M., Pintado, M. E., Malcata, F. X. 2005. Survival of probiotic bacteria in a whey cheese vector submitted to environmental conditions prevailing in the gastrointestinal tract. *International Dairy Journal* 15(6-9):921-927.
28. McFeeters, R. F., Barish, A. O. 2003. Sulfite analysis of fruits and vegetables by High-performance Liquid Chromatography (HPLC) with Ultraviolet Spectrophotometric Detection. *Journal of Agricultural and Food Chemistry* 51(6):1513-1517.
29. Miles, A. A., Mirsa, S. S., Irwin, J. O. 1938. The estimation of the bactericidal power of the blood. *Journal of Hygiene* 38 (06):732-749.
30. Pizzoferrato, L., Di Lullo, G., Quattrucci, E. 1998. Determination of free, bound and total sulphites in foods by indirect photometry-HPLC. *Food chemistry* 63(2):275-279.
31. Pambianchi, D. 2014. Benchmarking of $SO_2$ analysis instruments and methods in wine applications.

32. Plaza, A., Rumero, J., Silva, W., Morales, E., Torres, A., Aguirre, M. J. 2013. Extraction and quantification of $SO_2$ content in wines using a hollow fiber contactor. *Food Science and Technology International* 20(7):501-510.
33. Pitino, I., Randazzo, C. L., Mandalari, G., Lo Curto, A., Faulks, R. M., Le Marc, Y., Le., Bisignano, C., Caggia, C., Wickham, M. S. J. 2010. Survival of *Lactobacillus rhamnosus* strains in the upper gastrointestinal tract. *Food microbiology* 27(8):1121-1127.
34. Ruiz-Capillas, C., Jimenez-Colmenero, F. 2008. Determination of preservatives in meat products by flow injection analysis (FIA). *Food Additives & Contaminants* 25(10):1167-1178.
35. Roberts, A.C., McWeeny, D. J. 1972. The uses of sulphur dioxide in the food industry. *International journal of Food Science & Technology* 7(3):221-238.
36. Rossi, M., Amaretti, A., and Raimondi, S. 2011. Folate Production by Probiotics Bacteria. *Nutrients* 3(1):118-134.
37. Sousa, S., Pinto, J., Pereira, C., Xavier Malcata, F., Bertoldo Pacheco, M.T., Gomes, A. M., Pintado, M. 2015. In vitro evaluation of yacon (Smallanthus sonchifolius) tuber flour prebiotic potential. *Food and Bioproducts Processing* 95:96-105.
38. Silva, S. N. D. C. 2012. Analysis of the microbiological and antioxidant properties of dried fruit and leaf extracts of blueberry (Vaccinium corymbosum) [M.Sc. dissertation]. Escola Superior de Biotecnologia. 15-16pp. Universidade Católica Portuguesa; URI:10400.14/17585.
39. Takumi, K., de Jonge, R., Havelaar, A. 2000. Modelling inactivation of *Escherichia coli* by low pH: application to passage through the stomach of young and elderly people. *Journal of Applied Microbiology* 89:935-943.
40. Til, H.P., Feron, V.J. 1992. Toxicology of sulphting agents I: animal studies. *Food Additives and Contaminants* 9(5):587-595.
41. Vamanu, E. 2017. Effect of gastric and small intestinal digestion on lactic acid bacteria activity in a GIS1 simulator. *Saudi Journal of Biological Sciences* 24(7):1453-1457.
42. Wedzicha, B. L. 1984. Sulphur dioxide in foods– chemical interactions. Nutrition Bulletin 9(3):155-164.http://onlinelibrary.wiley.com/doi/10.1111/j.1467-3010.1984.tb01350.x/abstract
43. Walker, R. 1985 Sulphiting agents in foods: some risk/benefit considerations. *Food Additives and Contaminants* 2(1):5-24.
44. Wijnands, L. M., Pielaat, A., Dufrenne, J. B., Zwietering, M. H., van Leusden, F.M. 2009. Modelling the number of viable vegetative cells of *Bacillus cereus* passing through the stomach. *Journal of Applied Microbiology* 106(1):258-267.
45. Zavisic, G., Petricevic, S., Radulovic, Z., Begovic, J., Golic, N., Topisirovic, L., Strahinic, I. 2012. Probiotic features of two oral *Lactobacillus* isolates. *Brazilian Journal of Microbiology* 43(1):418-428.
46. Zhu, H., Hart, C. A., Sales, D., Roberts, N. B. 2006. Bacterial killing in gastric juice – effect of pH and pepsin on *Escherichia coli* and *Helicobacter pylori*. *Journal of Medical Microbiology* 55(9):1265-1270.